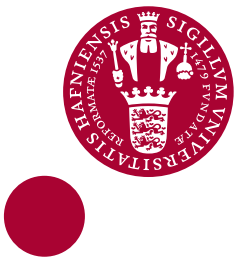

Glucosinolate structural diversity, identification, chemical synthesis and metabolism in plants

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Review

Glucosinolate structural diversity, identification, chemical synthesis and metabolism in plants

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ABSTRACT

The glucosinolates (GSLs) is a well-defined group of plant metabolites characterized by having an *S*-*β*-D-glucopyrano unit anomerically connected to an *O*-sulfated (*Z*)-thiohydroximate function. After enzymatic hydrolysis, the sulfated aglucone can undergo rearrangement to an isothiocyanate, or form a nitrile or other products. The number of GSLs known from plants, satisfactorily characterized by modern spectroscopic methods (NMR and MS) by mid-2018, is 88. In addition, a group of partially characterized structures with highly variable evidence counts for approximately a further 49. This means that the total number of characterized GSLs from plants is somewhere between 88 and 137. The diversity of GSLs in plants is critically reviewed here, resulting in significant discrepancies with previous reviews. In general, the well-characterized GSLs show resemblance to C-skeletons of the amino acids Ala, Val, Leu, Trp, Ile, Phe/Tyr and Met, or to homologs of Ile, Phe/Tyr or Met. Insufficiently characterized, still hypothetic GSLs include straight-chain alkyl GSLs and chain-elongated GSLs derived from Leu. Additional reports (since 2011) of insufficiently characterized GSLs are reviewed. Usually the crucial missing information is correctly interpreted NMR, which is the most effective tool for GSL identification. Hence, modern use of NMR for GSL identification is also reviewed and exemplified. Apart from isolation, GSLs may be obtained by organic synthesis, allowing isotopically labeled GSLs and any kind of side chain. Enzymatic turnover of GSLs in plants depends on a considerable number of enzymes and other protein factors and furthermore depends on GSL structure. Identification of GSLs must be presented transparently and live up to standard requirements in natural product chemistry. Unfortunately, many recent reports fail in these respects, including reports based on chromatography hyphenated to MS. In particular, the possibility of isomers and isobaric structures is frequently ignored. Recent reports are re-evaluated and interpreted as evidence of the existence of "isoGSLs", i.e. non-GSL isomers of GSLs in plants. For GSL analysis, also with MS-detection, we stress the importance of using authentic standards.

1. Introduction

1.1. Introduction to glucosinolates

Glucosinolates (GSLs) constitute a well-defined class of anionic natural products found in cabbages, mustards and related plants (mainly the "cabbage order", Brassicales), and share the potential to form isothiocyanates (ITCs) with the common structure R–N═C═S after hydrolysis (Fig. 1). The sharp tasting ITCs were historically known as mustard oils because molecules of this class are the pungent constituent in domestic mustard. All GSL structures are based on an *S*-*β*-D-glucopyrano unit anomerically connected to an *O*-sulfated (*Z*)-

Abbreviations: GSL, glucosinolate; dGSL, desulfoglucosinolate; ITC, isothiocyanate; OAT, oxazolidine-2-thione; t_R, retention time; 2D, two-dimensional; b.p., boiling point

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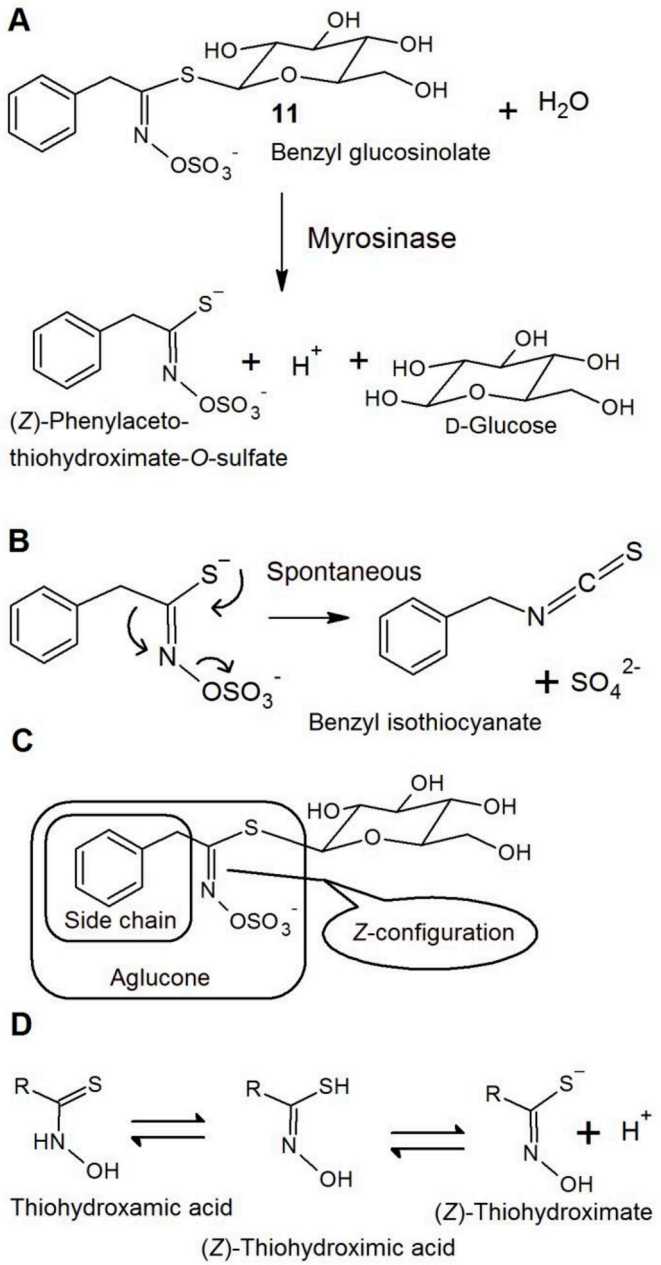

Fig. 1. A simple glucosinolate (GSL) and its enzymatic conversion to an isothiocyanate (ITC). (A) Structure of benzyl GSL and hydrolysis to the aglucone. (B) Spontaneous rearrangement of the aglucone to benzyl ITC. (C) Configuration of the thiohydroximate double bond and some common terms used in discussing GSL structures. (D) The relation of a thiohydroximate ion to thiohydroxamic and –imic acids.

thiohydroximate function. That is, a GSL is a thiohydroximate with a sulfate ester group at the oxygen and a glucose residue at the sulfur. The glucose residue is the pyranose form of D-glucose attached in *β*-linkage, i.e. a *β*-D-glucopyranoside. The rest of the GSL is referred to as the aglucone. The C═N double bond could potentially exist in two configurations. However, in GSLs this double bond has the *Z*-configuration, i.e. with the two heaviest atoms linked to the double bond on the same side (to the right when viewed as in Fig. 1).

While the above-described general structure seems to be invariable or almost so in nature, the part of the aglucone named the side chain (Fig. 1C) or "R-group" is subject to immense structural variation with associated biological consequences. Indeed, a focus of this review will be on variation of the side chain.

GSLs are generally hydrolyzed by plant enzymes, commonly known as myrosinases, which specifically hydrolyze the thioglucosidic bond (Section 4). The GSL structure and hydrolysis is most easily grasped by inspection of an example, such as the hydrolysis of one of the simplest known natural GSLs, benzyl GSL (**11**) (Fig. 1). Myrosinase-catalyzed hydrolysis of the thioglucosidic bond of **11** releases the sulfated thiohydroximate (Fig. 1A), which spontaneously rearranges to the ITC (Fig. 1B). Depending on the particular side chain, a different ITC will be formed. In contrast, hydrogen ion and sulfate will form from all GSLs, and glucose will form from most GSLs (substituted glucose is expected from a few). Many GSL analytical protocols take advantage of the ubiquitous products, including a general myrosinase assay following H^+ formation in a pH stat (Palmieri et al., 1987), visualization of myrosinase in gels by a pH indicator dye (Gonda et al., 2018), and determination of total GSLs from myrosinase-dependent glucose formation (VanEtten and Daxenbichler, 1977).

The rearrangement to ITC appears to be driven by loss of sulfate, leading to an electron-deficient N, recruitment of a bond from the side chain and simultaneous formation of a double bonded S using a sulfide lone pair (Fig. 1B). This 'Lossen-like' rearrangement was proposed after studies of chemical breakdown products of GSLs by Ettlinger and Lundeen (1956a), who went on to demonstrate the correct GSL structure by rational synthesis (Ettlinger and Lundeen, 1957). The structure, including (*Z*)-configuration, was confirmed by X-ray crystallography (Waser and Watson, 1963; Jaki et al., 2002).

The core structural element, a thiohydroximate, is the corresponding base of a thiohydroximic acid. Both are thiolo tautomers of the corresponding thiohydroxamic acid and thiohydroxamate (Fig. 1D) (e.g. Lemercier and Pierce, 2014). The early literature used the latter terms (with "a") for both tautomeric forms (Ettlinger et al., 1961). From the start of the GSL literature, it was noticed that the property of being a weak base makes the thiohydroximate-*O*-sulfate a good leaving group after the enzyme-catalyzed hydrolysis of the thioglucosidic bond, and explains the pH dependence of the final aglucone products (Ettlinger et al., 1961; Benn, 1977) (Section 4.3). Indeed, in the best understood myrosinase enzyme, from *Sinapis alba* L. (white mustard), there is no catalytic acid for assisting the aglucone departure (Burmeister et al., 1997, 2000). The fact that the GSL aglucone is an acid residue means that GSLs are not merely glycosides (acetals), but also have properties of esters. They share the susceptibility to hydrolytic attack at the anomeric C with glycosides, and the ability to liberate a good leaving group with esters. Even this extended classification of GSLs is simplified, due to the additional influences of the sulfate. The GSL functional group is unique.

1.1.1. Recommended nomenclature

Historically, the natural ITC-releasing glucosides were known as "mustard oil glucosides" of unknown exact structure (e.g. Greer, 1956). After the elucidation of the general GSL structure (Ettlinger and Lundeen, 1956a, 1957), a precise nomenclature was needed. The name "glucosinolate" was proposed in a Ph.D. thesis (Dateo, 1961) and used soon after in a scientific paper without explanation (Ettlinger et al., 1961). It became generally accepted around a decade later, after its use in a seminal review by Ettlinger and Kjær (1968). This nomenclature, explained below, is known as semisystematic nomenclature and is recommended in all scientific communication.

Dateo (1961) suggested naming a simple ion (with H replacing the side chain of **11**) "glucosinolate" (Fig. 2A) and using this backbone in derivative names (Fig. 2B–D). Hence, only derivatives of this exact backbone are by definition glucosinolates. The ion "glucosinolate" as defined by Dateo (1961) (Fig. 2A), has to our knowledge never been synthesized (Kjersgaard and Kjær, 1970) or discovered in nature.

In a very brief explanation, the name was derived from Greek "σιναπ-ελαιον" ("sinap elaion") supposed to mean mustard oil. The name "glucosinolate" would seem to signify a glucosylated, sulfated mustard oil precursor, with "sinol" signifying the mustard oil, "gluco"

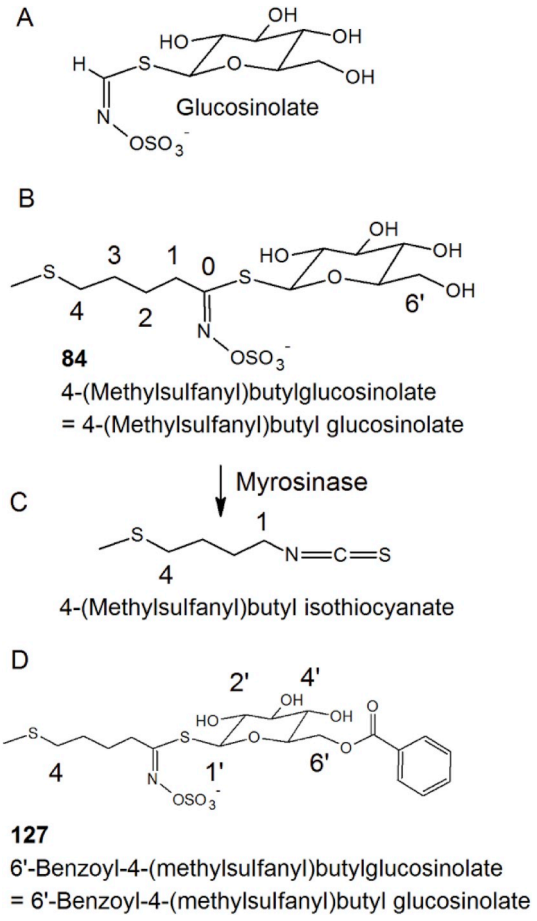

Fig. 2. Definition of the parent glucosinolate ion (A), and its use in naming natural derivatives (B, D), with numbering system indicated, using the originally proposed writing of names in one word (Dateo, 1961). The numbering system is retained in the isothiocyanate product (C). The modern practice by many authors of splitting glucosinolate anion names in two (given below each name) is followed in the rest of the text. Both practices are considered generally accepted.

the (thio)glucoside, and "ate" the oxyanion (the sulfate group) (Kjær and Thomsen, 1962a; Agerbirk and Olsen, 2012). The connection between the Greek term and the "o" in sinol was not explained, and it seems likely that Latin "*oleum*" (oil), or derived words in modern languages, has played a role in the reasoning (Agerbirk and Olsen, 2012). Indeed, similar names for ITCs have a long history; Gerhardt (1845) proposed the French term "sinapol sulfuré" for what was probably allyl ITC.

The GSL nomenclature of Dateo (1961) has gained universal acceptance; relatively simple names are obtained when the various GSLs are designated as derivatives of the GSL ion (Fig. 2). Biological variation of GSL structures is predominantly due to variation of the side chain (Fig. 3), but derivatives at the thioglucose moiety are also known (Fig. 2D). The numbering of GSL positions treats the first carbon in the side chain as number 1, meaning that the thiohydroximate carbon logically has number zero. This is practical when dealing with the rearranged ITC products, as R group names and numbering are hence identical in the GSL and corresponding ITC.

It follows from the definition (Fig. 1) that GSLs are anions. The names of the individual GSL anions were consistently written in one word, and still are by some authors (e.g., benzylglucosinolate, 4-hydroxyindol-3-ylmethylglucosinolate), with the salts written in two words (e.g. sodium benzylglucosinolate, potassium benzylglucosinolate) (Dateo, 1961; Ettlinger et al., 1961; Ettlinger and Kjær, 1968; Benn, 1977; Bak et al., 1999; Kiddle et al., 2001; Wittstock and Halkier, 2002; Agerbirk and Olsen, 2012; Wittstock et al., 2016). In general communication, many contemporary authors (e.g. Fahey et al., 2001; Halkier, 2016; Jeschke et al., 2016; Blažević et al., 2017) split names of the GSL anions in two words for easy reading (i.e. benzyl glucosinolate). An authoritative review even used a mixed practice (Halkier and Gershenzon, 2006). Evidently, GSL names in one or two words are synonyms in the actual scientific literature. [This is not generally the case in organic nomenclature; while phenyl acetate (CAS 122-79-2) is the ester of phenol and acetic acid, phenylacetate is the anion C_6H_5–CH_2–COO^- (Na^+ salt: CAS 114-70-5)]. In this review, names of free GSL anions will be written in two words, reflecting this practice by the apparent majority of modern authors. The shared GSL backbone is abbreviated "GSL" in structures (Fig. 3).

A popular model organism in plant biology is *Arabidopsis thaliana* (L.) Heynh. (thale cress, mouse-ear cress, or in plant molecular biology jargon simply "Arabidopsis", not italicized). In the *A. thaliana* field, a set of systematic name abbreviations for GSLs were used in an early paper (Brown et al., 2003), e.g. I3M for indol-3-ylmethyl GSL (**43**) and 4MOI3M for the 4-methoxy derivative (**48**). This preliminary set of abbreviations has achieved remarkable general acceptance. Even longer abbreviations such as "(*S*)2OH-2PE" (**40*S***) have been added to the system *ad hoc* in an attempt to cover more structures (Windsor et al., 2005). But extending this system to all GSLs, such as methylsulfonylalkyls and branched, oxidized aliphatics would seem impossible without re-inventing systematic organic nomenclature. A slightly simpler and extended system has been suggested, e.g. IM for **43** and BAR for **40*S*** (Olsen et al., 2016). For the limited number of GSLs in e.g. *A. thaliana* such abbreviations are useful, but the difficulties arising when expanded to further structures highlight the general usefulness of the semisystematic GSL nomenclature, which is itself an efficient abbreviation of alternative chemical names (Section 1.1.4). Further systems of nomenclature are briefly explained in Section 1.1.4., after presenting the biochemistry on which they depend.

In this review, we consistently use the semisystematic nomenclature first proposed by Dateo (1961) and the comprehensive numbering system first proposed by Fahey et al. (2001) and continued by Agerbirk and Olsen (2012) and Olsen et al. (2016). Selected structures and numbers are presented in this introductory section, while the entire known diversity is presented and illustrated in Section 2.

1.1.2. Biosynthesis

GSLs are biosynthesized from amino acids. In some cases, the amino acid precursor is obvious, such as Phe in the case of **11**, Val in the case of **56** and Ile in the case of **61** (Fig. 3). In these cases, the biosynthesis is relatively simple, consisting of multistep transformation of the amino acid to form the GSL. For example, benzyl GSL is biosynthesized in seven enzymatic steps from the standard amino acid Phe (Fig. 4). These steps are collectively known as the core biosynthesis, and were first suggested from classical tracer experiments (e.g. Underhill et al., 1962; Kutáček et al., 1962; Benn and Meakin, 1965; Underhill and Kirkland, 1972; reviewed by Ettlinger and Kjær, 1968; Mikkelsen et al., 2002).

In an elegant approach, the biosynthesis was confirmed by expressing amino acid activating genes from the initial step of cyanogenic glucoside biosynthesis in GSL-producing *A. thaliana*, resulting in accumulation of the corresponding GSLs (Bak et al., 1999; Wittstock and Halkier, 2002; Mikkelsen et al., 2002). This approach showed that conversion of amino acids to the corresponding oximes in GSL-containing plants will often lead to accumulation of the corresponding GSL, apparently due to a rather unspecific biosynthesis in the additional steps. Since then, an impressive international effort within the *A. thaliana* community and dominated by a few very active groups, has resulted in a very detailed understanding of the biochemistry and molecular biology of the biosynthesis of the GSLs in *A. thaliana* (Halkier and Gershenzon, 2006; Grubb and Abel, 2006; Sønderby et al., 2010a, 2010b; Halkier, 2016). Transfer of an entire functional GSL biosynthetic pathway to species not naturally producing GSLs is now possible (Geu-

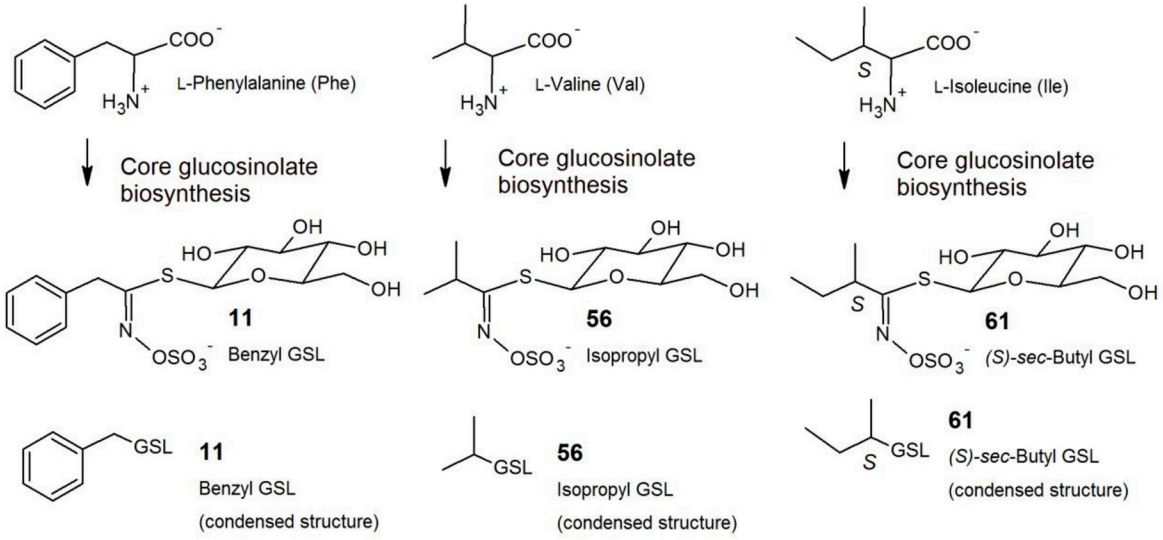

Fig. 3. Examples of glucosinolates derived directly from a standard amino acid precursor, and meaning of the abbreviation 'GSL' in condensed structures.

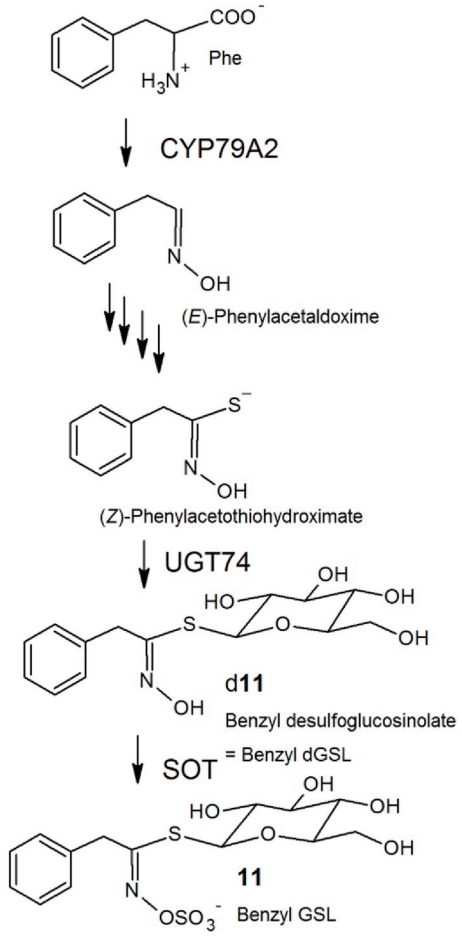

Fig. 4. Biosynthesis of benzyl glucosinolate from Phe (Wittstock and Halkier, 2000; Wittstock and Halkier, 2002; Mikkelsen et al., 2002). An oxime is the first intermediate, followed by a complex transformation to the thiohydroximate, and finally glucosylation and sulfation. By introduction of the sulfate group in the final step, the unstable intermediate that leads to isothiocyanate formation (Fig. 1) is avoided. The donor of the thioglucose S is glutathione (Schläppi et al., 2008; Geu-Flores et al., 2009a). The relevant (*E*)-oxime is the immediate product of the CYP79 (Clausen et al., 2015). In the further core biosynthesis not shown, the identity of one intermediate is yet uncertain (either an *aci*-nitro or a nitrile oxide functionality) (Sønderby et al., 2010a).

Flores et al., 2009b; Pfalz et al., 2011; Mikkelsen et al., 2012; Møldrup et al., 2012; Crocoll et al., 2016; Petersen et al., 2018).

The biosyntheses of some GSLs involves secondary modification after the core biosynthesis, such as modification of the side chain or glucose moiety. For example, after core biosynthesis of the parent GSL **43** from Trp, secondary modifications can occur, such as hydroxylations and further methylations (Fig. 5A). Further structural diversity is caused by chain elongation, consisting of elongation of the precursor amino acid before the core biosynthesis. Chain elongation is only well-established from a few precursor-amino acids (Section 2.4). In particular, the biosyntheses from Met and Phe/Tyr lead to a wide variety of GSLs due to effects of chain elongation before the core biosynthesis and secondary modifications happening after the core biosynthesis. For example, 2-hydroxybut-3-enyl GSLs (**24*R*** and **24*S***) and 4-benzoyloxybutyl GSL (**5**) in *A. thaliana* are biosynthesized from Met in such an elaborate way that the origin from Met is quite obscured in the final structures (Fig. 6).

Final complexities in GSL metabolism include relations to general metabolism (Kopriva and Gigolashvili, 2016), transport, and regulation. Transport of GSLs must happen between compartments in the cell (Halkier and Gershenzon, 2006; Grubb and Abel, 2006; Sønderby et al., 2010a, 2010b; Halkier, 2016) and between tissues and organs (Nour-Eldin et al., 2012; Halkier, 2016). All the processes are subject to fine-tuned spatiotemporal regulation (Burow, 2016; Burow and Halkier, 2017; Nintemann et al., 2018; Lee et al., 2018).

Biosynthesis of GSLs is mainly investigated in molecular detail from Phe, Trp, and Met (Sønderby et al., 2010a, 2010b), and additionally documented or anticipated from the following standard amino acids: Ala, Val, Leu, Ile, Glu, Tyr and in some cases their chain elongated homologs (Ettlinger and Kjær, 1968; Agerbirk and Olsen, 2012) (Section 2.4). For indirect biosynthesis from Trp, via the rare amino acid 4-methoxyindol-3-ylglycine, see below.

The biosynthetic pathways in most non-model plants are still poorly investigated, and their investigation is likely to be a future priority. As an example of the outcomes of studying other plants than *A. thaliana*, GSL biosynthesis from a non-standard amino acid was recently proposed with extensive evidence. The formerly unknown amino acid 4-methoxyindol-3-ylglycine appears to be the biosynthetic precursor of the unstable, rarely studied 4-methoxyindol-3-yl GSL (**[147]**), an intermediate in phytoalexin biosynthesis in *Brassica* spp. (Pedras and Yaya, 2013; Pedras et al., 2016). The above very unusual amino acid was shown by isotopic labeling experiments to be derived from indol-3-ylmethyl GSL (**43**) (Pedras and Yaya, 2013), which is ultimately derived

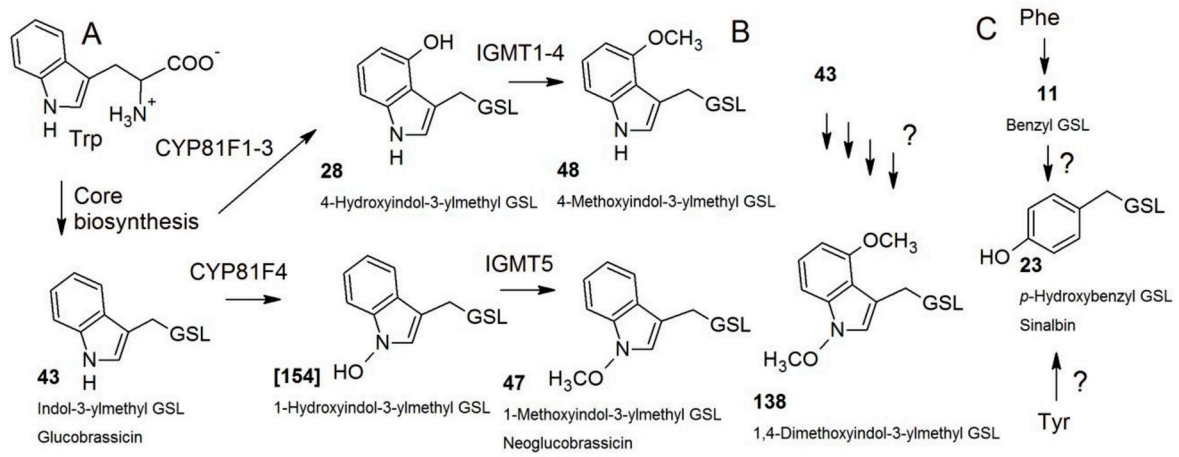

Fig. 5. Secondary modifications in glucosinolate (GSL) biosynthesis. (A) Biosynthesis of secondary modifications in Trp-derived GSLs ("indole GSLs") in *Arabidopsis thaliana* (Pfalz et al. 2011, 2016). (B) Biosynthesis of a more complex indole GSL is still unknown. (C) Two possible biosyntheses of *p*-hydroxybenzyl GSL (**23**), the relative importance of which in nature is not known. In the current paper, bold and square brackets around GSL numbers indicate incomplete MS and NMR-documentation for their existence (Section 2).

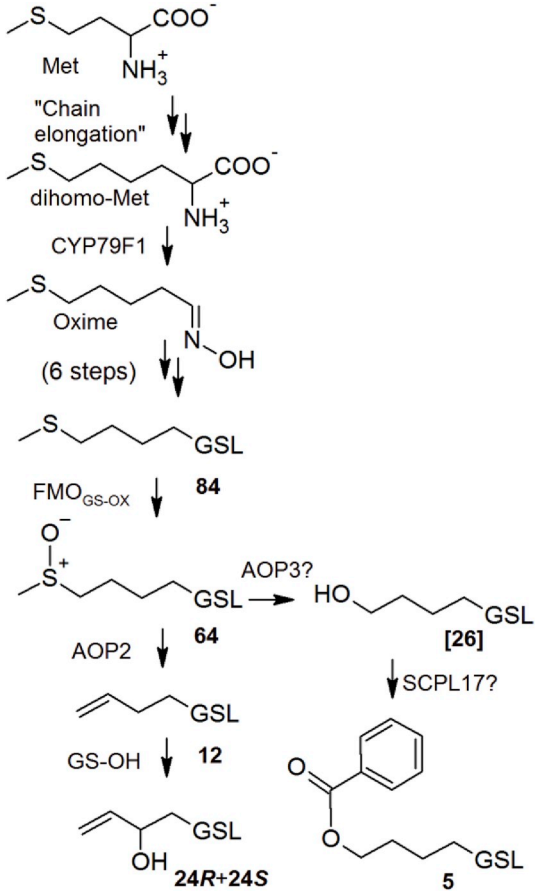

Fig. 6. Biosynthesis of six Met-derived glucosinolates (GSLs) in *Arabidopsis thaliana* starting with chain elongation (8 enzymatic steps for increasing the number of C atoms with two), followed by core GSL biosynthesis leading to the parent dihomoMet derived GSL **84**, 4-(methylsulfanyl)butyl GSL. Further sequential secondary modifications of the parent GSL, via 4-(methylsulfinyl)butyl GSL (**64**) and but-3-enyl GSL (**12**) ends with 2-hydroxybut-3-enyl GSL (mixture of two stereoisomers, **24*R*** and **24*S***, in this species) (Sønderby et al., 2010a). A different route leads from **64** to the alcohol **[26]** and the benzoyl ester **5** (Lee et al., 2012).

from Trp (Section 4.4.). The biosynthesis of other rare GSLs with side chains similar to that of Trp is unknown (Fig. 5B). In the related case of *p*-hydroxybenzyl GSL (**23**), biotechnological biosynthesis has been realized from Tyr (Bak et al., 1999). In natural plants, however, benzyl GSL (**11**) co-occurs with **23** in some but not all species (e.g. Bennett et al., 2004; Agerbirk et al., 2008), suggesting that the biosynthesis may in some cases originate from Phe, with the hydroxyl group added as a secondary modification (Fig. 5C).

From evolutionary arguments, the biosynthetic principles elucidated in model plants can be expected to be rather conserved in related species (Edger et al., 2018; Barco and Clay, 2019). However, convergent or parallel evolution (reviewed by Kliebenstein and Cacho, 2016) and mutation of enzyme specificity (Prasad et al., 2012, reviewed by Olsen et al., 2016) should be taken into account. Classical tracer experiments, of which many were performed by Canadian researchers at the Prairie Regional Laboratory in Saskatchewan, considered a different suite of species and GSLs than modern biochemistry currently does, establishing precursors and likely intermediates (Chisholm and Wetter, 1964; Underhill and Chisholm, 1964; reviewed by Ettlinger and Kjær, 1968). Some of these classical works have still not been revisited using modern molecular biology methods (Fig. 7). Early classical genetics using mainly *Brassica* species provided independent evidence for some of the secondary modifications, with one GSL leading to another, and the gene names used in *Brassica* are different from those in *A. thaliana* (Mithen, 2001; Velasco et al., 2017). Using sequence information from *A. thaliana*, many other species are now being investigated for homologs (e.g. van den Bergh et al., 2016; Zhang et al., 2016a; Katsarou et al., 2016; Wu et al., 2017; Byrne et al., 2017; Jeon et al., 2017), suggesting that our knowledge of GSL biosynthesis in non-model plants is about to be improved.

1.1.3. Catabolism by plant enzymes to isothiocyanates

The biological consequences of GSL structural variation are considerable, mainly due to the varied properties of the hydrolysis products. Many types of hydrolysis products are important and are discussed in Section 4. In this introductory section, only ITCs are discussed because of their central role in GSL biochemistry and structure elucidation. The significance of structural variation (Section 2) is well illustrated by eight domestic examples of GSLs and their corresponding ITCs known from widely used crops and foods (Fig. 8). Below, a superficial first glance is taken with focus on volatility and stability of ITCs and some effects on taste and flavor (for a more complete review of the impact of GSLs and ITCs on taste and flavor, see the recent review

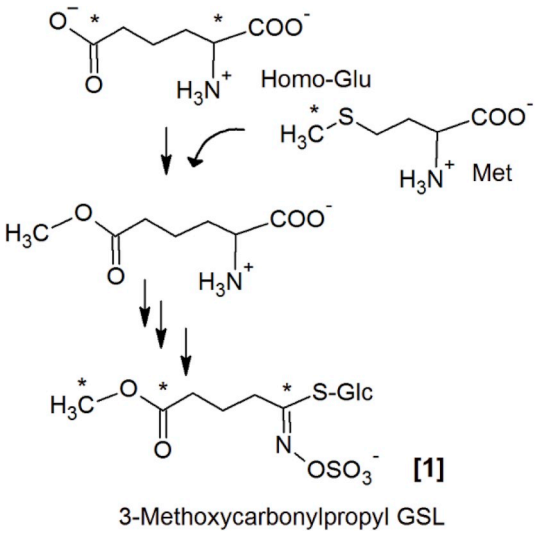

Fig. 7. Biosynthesis of the rare, unusual glucosinolate (GSL) 3-methoxycarbonylpropyl GSL (Kjær and Gmelin, 1957a) as evidenced by traditional tracer studies in an *Erysimum* sp. (Chisholm, 1973). Positions of radiolabeling are indicated with asterisks. Apart for the terminal methyl group derived from Met, the GSL was biosynthesized from homoGlu (probably derived from Glu), making it the only documented GSL biosynthesis from Glu. A more recent paper (Radulović et al., 2011) reported the free carboxylic acid, 3-carboxypropyl isothiocyanate from autolyzed *Erysimum diffusum* Ehrh. (diffuse wallflower), further supporting GSL biosynthesis from homoGlu in *Erysimum* spp. The brackets indicate the lack of modern spectroscopic confirmation of this structure, which is, however, based on very solid classical work.

by Bell et al., 2018). Methyl GSL (**51**, characteristic for capers, *Capparis* sp.) produces methyl ITC, a reactive, low boiling liquid (b.p. 109 °C). A somewhat less volatile, archetypical "mustard oil" is produced from benzyl GSL (**11**, from e.g. garden cress, *Lepidium sativum* L.), which produces benzyl ITC (b.p. 242 °C). Both are generally described as having a pungent flavor. In contrast, the *p*-rhamnosyloxy derivative (**110**, from e.g. the tropical "horseradish tree", *Moringa oleifera* Lam.) produces a non-volatile ITC, which is a solid in the pure state. No smell can possibly alert animals or humans to the reactive chemical nature of this compound (Müller et al., 2015), in contrast to the former two examples. However, **11** and other GSLs that are precursors of volatile ITCs can be present in the "horseradish tree" (Bennett et al., 2003; Fahey et al., 2018; Chodur et al., 2018). Those three cases exemplify how the side chain structure affects the general biological properties of hydrolysis products (Section 7), despite their shared ITC functionality. Wild accessions of *M. oleifera* were bitter and contained a unique derivative of **110**, to which the bitterness was ascribed (Chodur et al., 2018). Unfortunately, the GSL was misidentified (Section 2.7).

Three additional examples (Fig. 8), all from cabbages (*Brassica oleracea*), highlight the effects of modifying the side chain of Met-derived GSLs (Fig. 6). Whether the ITC from 4-(methylsulfinyl)butyl GSL (**64**) is sharp tasting is apparently not described in the literature (Bell et al., 2018). However, a sharp taste is expected due to the ITC functional group, while a smell is not expected due to the polar side chain. In contrast, the ITC from but-3-enyl GSL (**12**) is both sharp tasting, volatile and pungent, while the cyclic product (Fig. 9) from (*R*)-2-hydroxybut-3-enyl GSL (**24*R***) is bitter (Fenwick et al., 1983b; Beck et al., 2014).

Two further examples (Fig. 8) show how the side chain structure can further modify this class of degradation product. The examples are from two kinds of mustard seeds with contrasting aroma. The first example is *p*-hydroxybenzyl GSL (**23**, from e.g. white mustard, *S. alba*) that forms an unstable ITC with a reported half-life of few hours (Buskov et al., 2000), that hydrolyzes to an alcohol and thiocyanate ion. The contrasting example is allyl GSL (**107**, from e.g. black mustard, *Brassica nigra* (L.) K. Koch) that forms the more stable allyl ITC which is quite volatile (b.p. 151 °C) and is generally agreed to be responsible for the overwhelming sensation in the nose when ingesting e.g. pungent 'Dijon' mustard. The instability of *p*-hydroxybenzyl ITC explains why mustards prepared from white mustard seeds are mild-tasting in contrast to mustards prepared from black mustard seeds.

A GSL chemically related to **23** is indol-3-ylmethyl GSL (**43**) from e.g. cabbage. The ITC from this GSL is so unstable that it has never been isolated or even detected directly. Due to a fast reaction with ascorbic acid (Kiss and Neukom, 1966; Agerbirk et al., 1998), **43** degrades to a prominent metabolite in crushed cabbage, ascorbigen (Fig. 8). In fact, **43** was discovered in a search for the precursor of ascorbigen, formerly believed to be a native cabbage metabolite rather than a GSL degradation product formed during autolysis (Kutáček et al., 1962). In the absence of ascorbate, the unstable indol-3-ylmethyl ITC is hydrolyzed to thiocyanate ion and indol-3-ylmethanol. The unstable nature of **23** and **43** depends on activation of the benzylic carbon due to the side chain structure (Agerbirk et al., 2009).

As the last example, we consider 4-mercaptobutyl GSL (**133**), from the popular vegetable variously named "rucola", "arugola", "arugula", or "rocket" (*Eruca vesicaria* (L.) Cav.). It is often named *E. sativa* Mill. but this name is discouraged by www.theplantlist.org. The degradation of this GSL is complex. Two hydrolysis products are known, a dimeric ITC (Bennett et al., 2002) and a cyclic product (Fechner et al., 2018) (Fig. 8). The cyclic product is due to a direct intramolecular reaction of the monomeric ITC (Fechner et al., 2018). The dimeric ITC must be due to oxidative coupling, either before or after myrosinase-catalyzed hydrolysis (Cataldi et al., 2007), but the exact course of this reaction has not been unraveled. Hydrolysis before oxidation produces an unstable ITC: as the ITC is an electrophilic group and the mercapto group at the other end of the molecule is nucleophilic and well positioned for ring closure, a cyclic thionocarbamate results. It is currently not known how the peculiar sulfurous aroma (Pasini et al., 2011) of *E. vesicaria* depends on this complex chemical system. Similar cyclic products, derivatives of oxazolidine-2-thione (OAT), are known from GSLs with hydroxyl groups in the side chain at the *β*-position (e.g. (*R*)-2-hydroxybut-3-enyl GSL (**24*R***), Fig. 9).

1.1.4. Alternative nomenclature

Alternative nomenclature of GSLs is superfluous and rarely of much benefit. Many trivial names are ambiguous, and often several names are in use. Trivial names belong to a time when GSL structural diversity was considered limited. Leaders in the field, themselves having suggested most GSL trivial names, later suggested not using trivial names at all in scientific GSL work (page 100 of Ettlinger and Kjær, 1968). The objections were: lack of structural information, lack of definition (e.g. referring to anion or salt?) and non-logical "in" suffix. The topic is summarized here because improper use of trivial names can cause much inconvenience.

Many early structures were given trivial names (Fig. 9) that are still used too often. However, a dozen or so are in common use and useful to know. Most common names were proposed in the 1950s and 1960s, and were designed by combination of the prefix "gluco" with the common name of the corresponding ITC, which was in turn derived from the first plant in which it was identified. An example is glucoiberin (**73**, 3-(methylsulfinyl)propyl GSL), the precursor of iberin, 3-(methylsulfinyl) propyl ITC, first isolated from *Iberis amara* L. (bitter candytuft) (Kjær, 1960). In some cases, no ITC is known, and the GSL is named directly from the plant, e.g. glucobrassicin (**43**, indol-3-ylmethyl GSL) isolated from and named after the genus *Brassica* (Gmelin and Virtanen, 1961) (Fig. 5). A further discovered *N*-methoxy derivative was named neoglucobrassicin (Gmelin and Virtanen, 1962) (Fig. 5). However, when two additional structures (**28**, **48**) were discovered (Truscott et al., 1982a; 1982b), the authors wisely abstained from continuing this naming tradition. The name progoitrin is an early name that follows its own logic, being the precursor of goitrin (Greer, 1956) (Fig. 9). Two

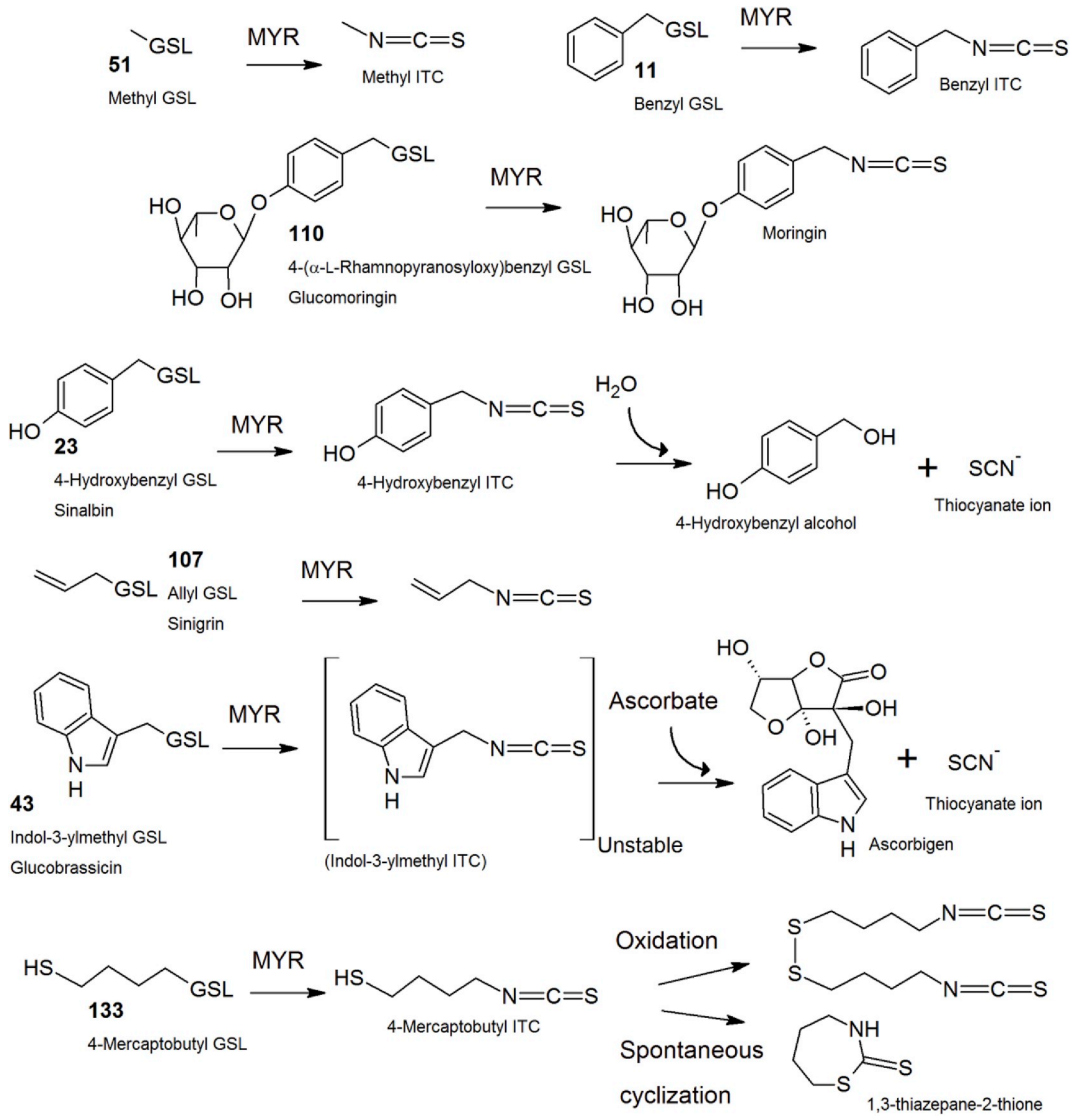

Fig. 8. Examples of the diversity of glucosinolate breakdown products. MYR, myrosinase.

ancient names, sinigrin and sinalbin (Fig. 9), are derived from "*Sinapis nigra*" (currently known as *B. nigra*) and *S. alba*, respectively. Phytochemistry and other dedicated natural product journals recommend proposed trivial names of novel GSLs to be derivatives of existing trivial names whenever possible. Examples are the derived trivial names homosinalbin (**140**) and 3,5-dimethoxysinalbin (**152**) proposed in recent years (Fig. 9). Suggestion of completely new GSL trivial names is rarely relevant.

In scientific communication, trivial names should always be accompanied by semisystematic names, also in abstracts and tables. Furthermore, the use of trivial names in scientific writing can only be justified if they are much simpler than the corresponding semisystematic names. For this reason, use of, e.g., glucoputranjivin for isopropyl GSL (**56**), glucotropaeolin for benzyl GSL (**11**) or glucocapparin for methyl GSL (**51**) are not reasonable, while the use of progoitrin for (*R*)-2-hydroxybut-3-enyl GSL (**24*R***) can be justified. Hence, trivial names should only be used as a last resort in case of GSLs with complex semisystematic names. The present authors furthermore propose limiting the use of trivial names to the dozen or so most well-known, and occasionally to GSLs of such structural complexity that the semisystematic name alone is extremely cumbersome (e.g. glucoisatisin for **121*R***, a 2,3-dihydro-2-oxoindol-3-ylacetate ester at the side chain oxygen of (*R*)-2-hydroxybut-3-enyl GSL) (Fig. 9).

Finally, although GSLs share properties with both esters and glucosides, they could also be named systematically as *S*-glucosides, e.g. benzyl GSL would be (*Z*)-benzylcarbothiohydroximate-*O*-sulfate *S*-*β*-D-glucopyranoside or (*Z*)-phenylacetothiohydroximate-*O*-sulfate *S*-*β*-D-glucopyranoside, using adapted hydroxamic acid nomenclature (IUPAC rule C 451.3). This is never seen in practice and is not recommended, but it illustrates the convenience of the semisystematic GSL nomenclature. Likewise, the nomenclature used for GSLs by Chemical Abstracts is of little relevance within the GSL community. In case of benzyl GSL (registered in CAS as the corresponding acid), the CAS name is *β*-D-glucopyranose, 1-thio-, 1-[*N*-(sulfooxy)benzeneethanimidate].

1.1.5. Classification

The most frequently used classification of GSLs is probably the distinction between "aliphatic", "aromatic" and "indole GSLs", three names wrongly used as synonyms of Met-, Phe- and Trp-derived GSLs, respectively. But that classification is of little biological and chemical significance and in conflict with the chemical meaning of the term "aromatic" (because the indole ring system is indeed aromatic) (Fig. 10). Below, we review meaningful classification criteria.

In discussions of biosynthesis, regulation, evolution and side chain skeleton structure (Section 2), a GSL classification on the basis of the amino acid precursor is meaningful, e.g. Trp-derived versus Ile-derived

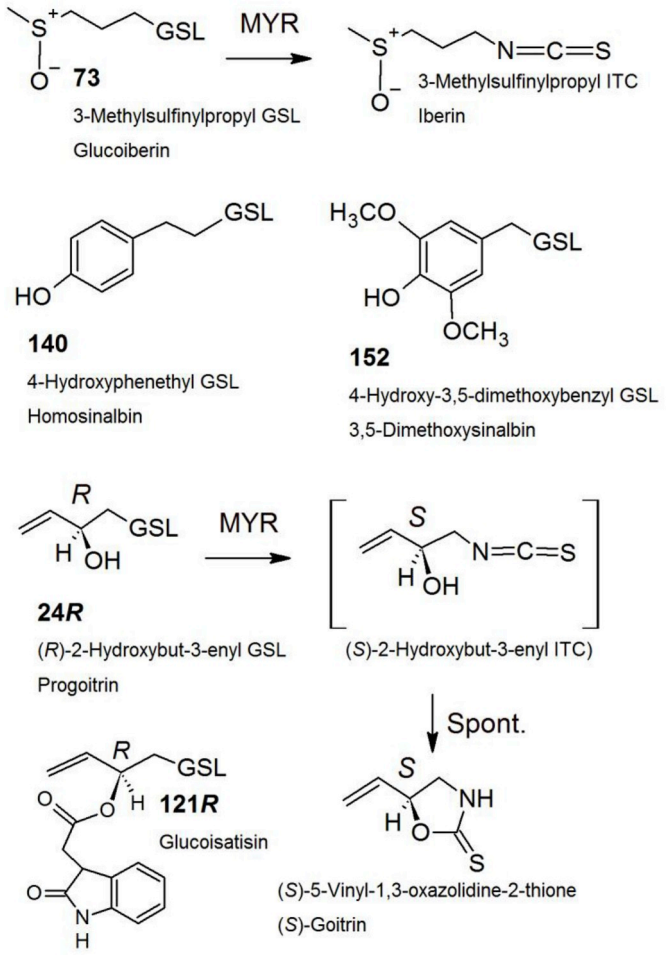

Fig. 9. Illustrative examples of trivial names of glucosinolates. MYR, myrosinase.

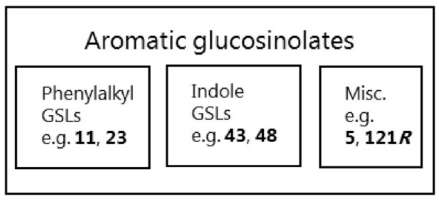

Fig. 10. Logical sub-divisions in the group of "aromatic glucosinolates", defined as glucosinolates containing one or more aromatic moieties.

versus Met-derived (Figs. 3–6). GSLs from the same biosynthetic group can be expected to show similar regulation (Section 1.1.2.), and a commonly observed evolutionary pattern is gain or loss of a precursor family of GSLs (Windsor et al., 2005; Agerbirk et al., 2008; Olsen et al., 2016).

Perhaps the most generally useful classification of GSLs is according to type of degradation product, which is useful in discussions of physiological and ecological effects of GSLs (Section 1.1.3.). At ITC-forming conditions, it is ecologically (e.g. Lazzeri et al., 2004) and nutritionally (e.g. Higdon et al., 2007; Ma et al., 2018) meaningful to distinguish at least three groups: stable ITC-yielding GSLs such as **11**, **51**, **73**, **110** and **140** versus thiocyanate ion-yielding such as **23**, **43** and **152** versus a third group of OAT-yielding such as **24*R*** and **24*S*** (Figs. 8, 9). Even stable ITCs show individual properties (e.g. anti-carcinogenic properties recently reviewed by e.g. Mitsiogianni et al. (2018), Soundararajan and Kim (2018); biofumigation properties tested by e.g. Neubauer et al. (2014), Sotelo et al. (2015)), presumably related to physico-chemical differences determining bioavailability (Holst and Williamson, 2004; Gimsing and Kirkegaard, 2009; Hanschen et al., 2014; Angelino and Jeffery, 2014). Hence, the first group could be further subdivided into GSLs yielding volatile versus nonvolatile ITCs or hydrophobic versus hydrophilic ITCs. In many species, additional types of GSL products and ITC products occur (Section 4), demanding more elaborate classification. In essence, the type of classification system should reflect the biological problem discussed.

The final classification criterion reviewed here is according to the presence or absence of an aromatic moiety in the GSL. This criterion is of little use, but can be meaningful in discussions of peak identification when using UV detectors. Relative to aliphatic GSLs, aromatic GSLs show additional absorption bands at higher wavelength and frequently exhibit local maxima and fine structure useful for identification (Section 5.1). A source of inspiration for the name is the "aromatic amino acids" (Phe, Tyr, and Trp), i.e. those with an aromatic moiety in the side chain, that are important for UV spectra of polypeptides. Similarly, we can speak of "aromatic GSLs" (i.e. with an aromatic moiety in the side chain) (Fig. 10). Subgroups of aromatic GSLs show characteristic UV spectra (Section 5). One subgroup of the aromatic GSLs is the indole GSLs, composed of **43** and several derivatives (Fig. 5). A large part of the remaining aromatic GSLs can be named phenylalkyl GSLs or "benzenic GSLs" (e.g. **11**, **23**, **140**). Some unusual substituted GSLs such as **5** and **121*R*** (Figs. 6 and 9) also fit in the group of aromatic GSLs.

1.2. Scope of the review

This review is meant not only for experts, but also for the novice in the field. Hence, we have aimed at introducing central topics in a way suitable for young scientists and newcomers with expertise in other fields. The central objective of this review is to provide a critical, comprehensive overview of documented GSL structural variation in plants, including classical work. A supplemental goal is to provide an up-to-date review of biochemical effects of GSL structural variation and related analytical and synthetic tools. However, in the latter cases, we focus on the past decades rather than an exhaustive treatment.

Critical, comprehensive reviews of GSL structural variation are surprisingly few considering the popular status of GSL research. The early literature was discussed elsewhere (Agerbirk and Olsen, 2012), but two authoritative early reviews should be mentioned: Kjær (1960) listing 30 characterized natural ITCs corresponding to 30 GSLs from plants, and Ettlinger and Kjær (1968) listing 50 directly or indirectly characterized natural GSLs. In the following three decades, no thorough, critical structural review was published. Hence, the publication in 2001 of a comprehensive, but only partially critical GSL review by Fahey et al. (2001) was a milestone, providing a useful table of GSLs reported in each plant species. However, multiple plain errors, lack of critical evaluation of the literature cited, and systematic problems in the interpretation of the data behind the table limited its value. For instance, systematic interpretation of detected thiocyanate ion as *p*-hydroxybenzyl GSL (**23**) was incorrect, as this ion can also signify a number of other GSLs, including indole GSLs (Fig. 8). Nevertheless, the Fahey et al. (2001) review provided a first attempt of a much-needed update on reported GSL structural diversity, listing 120 suggested structures numbered **1–120**. Since then, only one comprehensive, critical review on GSL structural diversity has been published (Agerbirk and Olsen, 2012), with a subsequent corrigendum (Agerbirk and Olsen, 2013). In that review, including the corrigendum, an additional 26 well-characterized GSLs were identified from the period after the Fahey et al. (2001) review (numbered **121–143**) [with some numbers including two diastereomers, e.g. **121*R***, **121*S***], and one additional structure (**40*R***) was identified from the period before the year 2000. However, from unsystematic scrutiny of the numbers up to 120, no less than 15 structures could immediately be abandoned as never characterized or documented, with the lack of data attributed to

misinterpretation, double-registration, obviously insufficient characterization, claims based on data not shown or in several cases a complete lack of evidence (Agerbirk and Olsen, 2012).

In section 2 of the present review, we attempt to compile all the structures satisfactorily characterized from 2011 to mid-2018. Furthermore, we systematically re-evaluate all previously identified structures from the reviews by Fahey et al. (2001) and Agerbirk and Olsen (2012). From this combined analysis, we aim to provide a complete list of documented, characterized GSLs up to mid-2018. For maximum coherence with the previous literature, the numbering system initiated by Fahey et al. (2001) is continued.

Sometimes only partial structural elucidation of GSLs is obtained. Acknowledging this fact, we furthermore provide an extensive (but not comprehensive) list of GSLs that have been reported with less than full structural information, yet sufficient for recognizing that a novel structure is at hand. Cases where one specific structure seems likely are discussed in Section 2.5, while cases where several structures all seem likely are discussed in section 2.7. Due to the massive amount of preliminary MS data published over recent years, the latter group can only be represented with a small subset of those suggested GSLs based on poor evidence.

Botanical nomenclature follows www.theplantlist.org, but in many cases synonyms, as used in the cited literature, are also given as a supplement.

The following sections each illustrate some feature or aspect of GSL structures and their chemical variation and properties. Relevant reviews will be referred to in each section. A large number of recent, excellent specialized reviews cover many other aspects of GSLs, including volumes edited by Kopriva (2016) and by Mérillon and Ramawat (2017).

2. Glucosinolate structures from plants

The list of reported GSLs and the degree of structural characterization (Table 1) essentially represents a continuum, ranging from the only two natural GSLs characterized by X-ray crystallography (**73**, **107**), to GSLs never characterized as such but only inferred from full or even tentative identification of their ITCs. For this review, it was deemed relevant to provide a simple indication of the strength of the evidence. The indication had to be objective and based on documented results only. In other words, the indication should neither take into account the reputation of e.g. the senior author nor reference to "results not shown". As ITCs may be formed independently of GSLs (Section 2.1.), the criterion had to be based on the evidence presented for the actual GSLs (or dGSLs), as opposed to ITCs or other aglucone breakdown products. Finally, the criterion should preferably be simple. We decided on just two groups, insufficiently characterized (compound numbers consistently shown in brackets) and sufficiently characterized (shown as plain compound numbers), and to base the grouping on the main criterion in contemporary dedicated natural product journals: the availability of extensive spectroscopic evidence, in particular NMR and MS data.

We stress that this distinction is meant as a guide and incentive for scientific progress, not in any way as a criticism of past or present authors, and we acknowledge that future reviewers may well decide other criteria as suitable (e.g. a combination of spectroscopic and enzymatic evidence or confirmation by synthesis). Indeed, structures suggested by some of the present authors and of generally esteemed past leaders in the field fall into both groups. However, a further particular advantage of NMR and MS as criteria for accepting GSLs is that these results can be reported in sufficient detail and objectivity to be critically evaluated by the reader, and even challenged (Section 2.7.).

Before making a choice, more comprehensive criteria were considered but abandoned. Essentially, it was neither meaningful nor objective to grade the relative strength of e.g. biosynthetic evidence, the highly variable kinds of enzymatic and wet chemistry procedures used in both historical and present day papers, and sophisticated recent arguments based on complex organic chemistry experiments dealing with apparently unstable GSLs (e.g. **[147]**).

Applying the simple "NMR + MS" criterion seemed to provide a meaningful division of well-established GSLs (Section 2.4.) from a heterogeneous group of less well-established or even questionable GSLs. The second group was very diverse, ranging from GSLs that were very sparsely characterized to some that were extensively characterized in historical reports (indirectly or even directly, e.g. **[1]**), but had apparently never been revisited since (Section 2.5.). In a few cases where the simplified criterion appeared unfair to particularly extensive classical or recent challenging experimental work, an explanatory note was added to the list, describing the particular merits of alternative or classical kinds of evidence (Table 1). However, even in these cases, which frequently involved GSLs of quite atypical structure, we consider modern spectroscopic reexamination to be of relevance. As a guide to the classical methods used by many early authors, we present an overview of past and present methods in section 2.1., and introduce the key NMR technique as applied to GSL identification in Section 2.2.

During our search into the documentation of the generally accepted GSLs from the review by Fahey et al. (2001), we realized that surprisingly many were not scientifically reported at all. We discontinued those associated with misunderstandings or obviously insufficient characterization or documentation (e.g., result not shown). The continued mentioning in the literature of such undocumented claimed GSLs also relates to intellectual property rights. For example, an apparently fictitious GSL, "2-methyl-2-propenyl GSL" (CAS 956700-01-9), listed as "60" in a past review (Table 1), is linked to two patents according to a SciFinder search. Also in this aspect, it seems important to uphold rigorous scientific standards in dealing with structural GSL diversity.

2.1. Methods used for glucosinolate structure characterization since 1950

Efficient chromatographic separation and identification of intact GSLs was historically difficult, and in many cases, GSLs have been deduced indirectly, from structure elucidation of breakdown products or derivatives.

The field of GSL diversity virtually exploded when the innovative technique of paper chromatography was introduced (Kjær, 1960). GSLs could be isolated this way, and then characterized by products of chemical breakdown (Bones and Rossiter, 2006), and their sensitivity to myrosinase-catalyzed hydrolysis (e.g. Kjær and Gmelin, 1957b). The breakdown products were isolated in the same new way and structurally identified by classical natural product techniques. In the case of β-hydroxyl GSLs, isolation and identification of cyclic OAT products served the same purpose (Fig. 9). This combination of evidence was strong because GSLs themselves were characterized and, at the time, accepted as conclusive even though dedicated present day journals would require NMR and MS. Even some early NMR data (Kjær and Thomsen, 1962b; Chisholm, 1973) have been deemed historical (Table 1) due to the low NMR frequencies available then. In several cases (e.g. **40*S***, **73**), GSLs inferred in this way ("isol + MYR" in Table 1) have subsequently been confirmed by spectroscopic structure elucidation of the intact GSL, while in other cases a modern follow-up is still lacking (**[1]**, **[9]**, **[14]**, **[113]**).

Isolation of ITC products or derivatives after myrosinase treatment of a crude GSL preparation or a crude extract is a less conclusive procedure as this does not allow confirmation of the actual GSL. This is still the only kind of evidence behind a range of γ-hydroxyl GSLs, e.g. **[33]**-**[37]**. The introduction of GC-MS was another milestone, leading to (sometimes only tentative) identification of some minor ITCs (e.g., **[20]**, **87**). In such cases, the corresponding GSLs were typically not isolated first. When MS and high-resolution NMR data are given, the present reviewers accept this method for identification of a natural ITC, but will regard the deduction of the corresponding GSL as tentative, because ITCs can be formed from other precursors than GSLs. Such

Table 1
Glucosinolates satisfactorily characterized to be naturally biosynthesized in plants (numbers in bold and without brackets, Section 2.4.), as well as some partially characterized structures [in brackets] with reasonable evidence for one single structure (Section 2.5.) and a few that might be considered artificial (labeled "Art") (Section 2.6.). The division in satisfactorily and partially characterized structures was based on availability of NMR as well as MS evidence for the GSL or dGSL, although other criteria would also be meaningful as further discussed in the text (Section 2.1.) and notes for **[1]**, **[3]**, **[26]**, **[42]**, **[113]**, **[144]**-**[147]**, **[154]**.

No	Class[a]	Semisystematic name(s) (Trivial name)[b]	First documented report/species[c]	Original and/or selected later critical evidence[d]
[1]	Glu	3-Methoxycarbonylpropyl GSL (Glucoerypestrin)	Kjær and Gmelin (1957a)/*Erysimum rupestre* = *E. pulchellum*	OrRep: isol + MYR, tetraacetate of GSL (OptRot, elem. comp., IR), ITC (compared with synth. ref.), TU (elem. comp., UV, IR); GSL: partial 100 MHz NMR (Chisholm, 1973) [Note: from an analytical chemistry point of view still needs verification, although the evidence is strong]
[2]	Trp	1-Acetylindol-3-ylmethyl GSL *N*-Acetylindol-3-ylmethyl GSL (*N*-Acetylglucobrassicin)	Schraudolf and Bäuerle (1986)/*Tovaria pendula*	OrRep: dGSL (EI-MS) [Note: This level of evidence is low. An independent paper claimed confirmation of a dGSL from this plant of expected *m/z* value (Mithen et al., 2010) (results not shown except for an HPLC peak)]
[3]	Phe	4-(4′-*O*-Acetyl-α-L-rhamnopyranosyloxy)benzyl GSL (4-Acetylglucomoringin)	Kjær et al. (1979)/*Moringa peregrina*	OrRep: ITC (IR, MS, NMR); GSL: MS (Bennett et al., 2003; Waterman et al., 2014); ITC: MS (Waterman et al., 2014); NIT: UV, MS, NMR (Faizi et al., 1994) [Note: from an analytical chemistry point of view still needs verification, although the evidence is strong]
4	Phe	2-(α-L-Arabinopyranosyloxy)-2-phenylethyl GSL	Olsen et al. (1981)/*Sesamoides canescens* = *S. interrupta* and *S. pygmaea* = *Reseda phyteuma*	OrRep: GSL (NMR), NIT (NMR) [Note: C2 absolute configuration is not resolved]
5	Met	4-(Benzoyloxy)butyl GSL	Hogge et al. (1988)/*Arabidopsis thaliana*	OrRep: dGSL (MS), ITC (MS); GSL: MS (Bringmann et al., 2005); dGSL: UV, MS, NMR (Graser et al., 2001; Reichelt et al., 2002)
[6]	Met	2-(Benzoyloxy)ethyl GSL	Daxenbichler et al. (1991)/*Arabidopsis thaliana*, *Moricandia arvensis*	OrRep: ITC (MS); GSL: MS (Bringmann et al., 2005)
[7]	Ile	(1*R*)-1-(Benzoyloxymethyl)propyl GSL (Glucobenzsisaustricin)	Kjær and Christensen (1962b)/*Sisymbrium austriacum*	OrRep: TU (and OAT and benzoic acid after alkaline hydrolysis)
No. "8" listed in Fahey et al. (2001) is discontinued, since the citation from Daxenbichler et al. (1991) of "benzoyloxymethyl GSL" has no basis in the paper. Possible misreading of Pr as Ph in the formula $PrCO_2Me$.				
[9]	Val	(1*R*)-2-Benzoyloxy-1-methylethyl GSL (Glucobenzosisymbrin)	Kjær and Christensen (1961)/*Sisymbrium austriacum*	OrRep: isol + MYR (Glc, ITC), wet chem., TU (UV, IR, compared with synth. ref.)
[10]	Met	3-(Benzoyloxy)propyl GSL (Glucomalcolmiin)	Kjær and Gmelin (1956b)/*Malcolmia maritima*	OrRep: isol + MYR (SO_4^{2-}, Glc, ITC), TU (elem. comp., UV, IR); GSL: MS (Bringmann et al., 2005); dGSL: UV, MS (Hogge et al., 1988; Reichelt et al., 2002); ITC: MS (Hogge et al., 1988); NIT: MS (Spencer and Daxenbichler, 1980)
11	Phe	Benzyl GSL (Glucotropaeolin)	Gadamer (1899a), 1899b/*Tropaeolum majus*	GSL: MS, MS2 (Maldini et al., 2017), NMR (Ibrahim et al., 2018); dGSL: UV, MS, MS2 (Kusznierewicz et al., 2013), NMR (Agerbirk et al., 2008; Ibrahim et al., 2018); TMSi of dGSL: EI-MS, CI-MS (Christensen et al., 1982); ITC: MS (Blažević and Mastelić, 2009)
12	Met	But-3-enyl GSL (Gluconapin)	Ettlinger and Hodgkins, 1955; Kjær et al. (1953)/*Brassica napus*	GSL: MS, MS2 (Maldini et al., 2017), NMR (Ibrahim et al., 2018); dGSL: UV, MS, MS2, NMR (Zimmermann et al., 2007; Olsen et al., 2016; Ibrahim et al., 2018); TMSi of dGSL: EI-MS, CI-MS (Christensen et al., 1982); ITC: MS (Vaughn and Berhow, 2005)
[13]	?	*n*-Butyl GSL	Kjær and Schuster (1971)/*Capparis flexuosa* = *Cynophalla flexuosa*	OrRep: TU from MYR product of mixed GSL preparation; ITC: MS (Kojima et al., 1973) [Note: various later claims from HPLC-MS do not present evidence for *n*-butyl GSL due to possible isomers (such as in Barillari et al., 2005b)]
[14]	Phe/Tyr	3,4-Dihydroxybenzyl GSL (Glucomatronalin)	Danielak and Borkowski (1970)/*Hesperis matronalis*	OrRep: isol + MYR (SO_4^{2-}, Glc), GSL (IR, UV); GSL: MS (Bennett et al., 2004); TMSi of dGSL: EI-MS, CI-MS (Boufford et al., 1989)

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Table 1 (*continued*)

No	Class[a]	Semisystematic name(s) (Trivial name)[b]	First documented report/species[c]	Original and/or selected later critical evidence[d]
15	Phe/Tyr	3,4-Dimethoxybenzyl GSL	Ettlinger et al. (1966)/*Heliophila longifolia* = *H. coronopifolia*	OrRep: TU (UV, IR, MS, compared with synth. ref.); dGSL: UV, MS, MS2, NMR (Pagnotta et al., 2017); ITC: MS (De Nicola et al., 2012; Pagnotta et al., 2017); NIT: MS (Pagnotta et al., 2017)
[16]	?	Ethyl GSL (Glucolepidiin)	Kjær and Larsen (1954)/*Lepidium menziesii* = *L. virginicum* subsp. *menziesii*	OrRep: TU (PC, IR and mixed melting point compared with auth. ref.; TU derived from MYR product of defatted seed meal)
No. "17" listed in Fahey et al. (2001) was discontinued (Agerbirk and Olsen, 2012), since it was a synonym of **30**.				
No. "18" listed in Fahey et al. (2001) ("hept-6-enyl GSL") was discontinued (Agerbirk and Olsen, 2012) due to insufficient evidence.				
No. "19" listed in Fahey et al. (2001) ("hex-6-enyl GSL") was discontinued (Agerbirk and Olsen, 2012) due to insufficient evidence.				
[20]	?	*n*-Hexyl GSL	Kojima et al. (1973)/*Wasabia japonica* = *Eutrema japonicum*. Disputed by (Kjær et al., 1978)/*Raphanus sativus* = *R. raphanistrum* subsp. *sativus*	ITC (GC-MS compared with auth. ref.) (OrRep, Kjær et al., 1978) [Note: various later claims from HPLC-MS do not present evidence for *n*-hexyl GSL due to possible isomers]
No. "21" listed in Fahey et al. (2001) ("2-hydroxybenzyl GSL") is discontinued, since no report was found. A later semisynthetic preparation (Bennett et al., 2004) from **109** using glycosidase treatment does not represent a natural GSL. Existence as biosynthetic intermediate of **109** seems possible.				
22	Phe	3-Hydroxybenzyl GSL (Glucolepigramin)	Friis and Kjær (1963)/*Lepidium graminifolium*	GSL: MS (Bennett et al., 2004); dGSL: MS, MS2, NMR (Pagnotta et al., 2017); NIT: MS (Pagnotta et al., 2017)
23	Phe/Tyr	4-Hydroxybenzyl GSL (Sinalbin, Glucosinalbin)	Gadamer (1897a)/*Sinapis alba*	GSL: MS (Bennett et al., 2004), UV, IR, NMR (Fabre et al., 1997; Ibrahim et al., 2018); dGSL: UV, MS, MS2, NMR (Agerbirk et al., 2008; Kusznierewicz et al., 2013; de Graaf et al., 2015; Olsen et al., 2016); TMSi of dGSL: EI-MS, CI-MS (Christensen et al., 1982); ITC: MS (Spencer and Daxenbichler, 1980; Vaughn and Berhow, 2005); NIT: MS (Spencer and Daxenbichler, 1980; Vaughn and Berhow, 2005)
24*R*	Met	(2*R*)-2-Hydroxybut-3-enyl GSL (Progoitrin)	Greer (1956); Schultz and Wagner (1956b)/*Brassica napus*	GSL: MS, MS2, MS3, (Maldini et al., 2017); NMR (Ibrahim et al., 2018); dGSL: UV, MS, MS2, MS3, NMR (Agerbirk et al., 2008; Kusznierewicz et al., 2013; Olsen et al., 2016; Ibrahim et al., 2018); TMSi of dGSL: EI-MS, CI-MS (Christensen et al., 1982); NIT: MS (Spencer and Daxenbichler, 1980); OAT: MS, OptRot (Daxenbichler et al., 1965; Spencer and Daxenbichler, 1980)
24*S*	Met	(2*S*)-2-Hydroxybut-3-enyl GSL (Epiprogoitrin)	Daxenbichler et al. (1965)/*Crambe abyssinica* = *C. hispanica* subsp. *abyssinica*	GSL: MS, MS2, MS3 (Millán et al., 2009); NMR (Ibrahim et al., 2018); dGSL: UV, MS, MS2, MS3, NMR (Kusznierewicz et al., 2013; Olsen et al., 2016; Ibrahim et al., 2018); NIT: MS (Spencer and Daxenbichler, 1980); OAT: MS, OptRot (OrRep; Spencer and Daxenbichler, 1980)
[25]	Met	3-Hydroxybutyl GSL	Kjær and Schuster (1971)/*Capparis flexuosa* = *Cynophalla flexuosa*	OrRep: TU, THOT [Note: absolute configuration not resolved]
[26]	Met	4-Hydroxybutyl GSL	Kjær and Schuster (1971)/*Capparis flexuosa* = *Cynophalla flexuosa*	OrRep: TU compared with synth. ref.; GSL: MS (Bringmann et al., 2005); dGSL: MS (Hogge et al., 1988); ITC: MS (Hogge et al., 1988) [Note: Although not itself conclusively identified, this suggested GSL is a required intermediate in the current model (Sønderby et al., 2010a, 2010b) for the biosynthesis of **5** from **64**, both conclusively identified]
27	Met	2-Hydroxyethyl GSL	Hu et al. (1989)/*Capparis masaikai* = *C. sikkimensis* subsp. *masaikai*	OrRep: GSL (NMR), OAT (MS, NMR)
28	Trp	4-Hydroxyindol-3-ylmethyl GSL (4-Hydroxyglucobrassicin)	Truscott et al. (1982a)/*Brassica napus* and *B. oleracea*	OrRep: TMSi of dGSL (UV, EI-MS, CI-MS, NMR); GSL: MS, MS2, MS3 (Millán et al., 2009; Maldini et al., 2017); dGSL: UV, MS, NMR (Kiddle et al., 2001; Agerbirk et al., 2001b), MS2 (Pfalz et al., 2016);
29*S*	Ile	(2*S*)-2-Hydroxy-2-methylbutyl GSL (Glucocleomin)	Kjær and Thomsen (1962a)/*Cleome spinosa*	OrRep: OAT (UV, IR, MS, NMR) (Christensen and Kjær, 1963a); dGSL: NMR (Ibrahim et al., 2018); NIT: MS (Spencer and Daxenbichler, 1980; Songsak and Lockwood, 2002)

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No	Class[a]	Semisystematic name(s) (Trivial name)[b]	First documented report/species[c]	Original and/or selected later critical evidence[d]
30	Ile	(1*R*)-1-(Hydroxymethyl)propyl GSL (Glucosisaustricin)	Kjær and Christensen (1962a)/*Sisymbrium austriacum*	OrRep: isol + MYR (SO_4^{2-}, Glc), OAT (PC compared with synth. ref., OptRot); dGSL: MS, MS2, NMR (Agerbirk et al., 2010a; Olsen et al., 2016)
31	Leu	2-Hydroxy-2-methylpropyl GSL (Glucoconringiin)	Kjær et al. (1956b)/*Conringia orientalis*	GSL: MS, NMR (Bennett et al., 2004; Olsen and Sørensen, 1979); TMSi of dGSL: EI-MS, CI-MS (Boufford et al., 1989); NIT: MS (Spencer and Daxenbichler, 1980); OAT: MS (Spencer and Daxenbichler, 1980)
[32]	Met	3-Hydroxy-6-(methylsulfinyl)hexyl GSL	Kjær and Schuster (1973)/*Erysimum rhaeticum*	OrRep: ITC, THOT [Note: C3 and S absolute configuration is not resolved]
[33]	Met	3-Hydroxy-5-(methylsulfinyl)pentyl GSL	Kjær and Schuster (1970)/*Erysimum hieracifolium* = *E. odoratum*	OrRep: THOT (UV, IR, MS) [Note: C3 and S absolute configuration is not resolved]
[34]	Met	3-Hydroxy-6-(methylsulfonyl)hexyl GSL	Kjær and Schuster (1973)/*Erysimum rhaeticum*	OrRep: ITC, THOT [Note: C3 absolute configuration is not resolved]
[35]	Met	3-Hydroxy-5-(methylsulfonyl)pentyl GSL	Kjær and Schuster (1970)/*Erysimum hieracifolium* = *E. odoratum*	OrRep: ITC (UV, IR, MS, NMR), THOT (UV, IR, MS) [Note: C3 absolute configuration is not resolved]
[36]	Met	3-Hydroxy-6-(methylsulfanyl)hexyl GSL	Kjær and Schuster (1973)/*Erysimum rhaeticum*	OrRep: THOT (UV, IR, MS) [Note: C3 absolute configuration is not resolved]
[37]	Met	3-Hydroxy-5-(methylsulfanyl)pentyl GSL	Kjær and Schuster (1970)/*Erysimum hieracifolium* = *E. odoratum*	OrRep: THOT (UV, IR, MS, NMR), TU (UV, IR, MS, NMR) [Note: C3 absolute configuration is not resolved]
38*S*	Met	(2*S*)-2-Hydroxypent-4-enyl GSL (Gluconapoleiferin)	Tapper and MacGibbon (1967)/*Brassica napus* and *B. campestris* = *B. rapa*	OrRep: OAT (UV, IR, NMR); GSL: MS, MS2, MS3 (Millán et al., 2009); dGSL: MS (Ishida et al., 1997); TMSi of dGSL: EI-MS, CI-MS (Christensen et al., 1982; Shaw et al., 1989); OAT: MS (Spencer and Daxenbichler, 1980); [Note **38*S*** → OAT with (*S*)-configuration. The hypothetic epimer "**38*R***" is expected but not documented]
No. "39" listed in Fahey et al. (2001) ("2-hydroxypentyl GSL") was discontinued due to insufficient evidence (Agerbirk and Olsen, 2012). Claimed ITC unexpected; an OAT would be expected.				
40*R*	Phe	(2*R*)-2-hydroxy-2-phenylethyl GSL (Epiglucobarbarin)	Gmelin et al. (1970)/*Sibara virginica* = *Planodes virginicum*	OrRep: OAT (OptRot); GSL: MS (Bennett et al., 2004), NMR (Agerbirk et al., 2001a); dGSL: UV, MS, MS2, MS3, NMR (Huang et al., 1994; Agerbirk et al., 2001a; Agerbirk et al., 2015a; Olsen et al., 2016: Fig. 14); OAT: UV, IR, MS, NMR, OptRot (Agerbirk and Olsen, 2015) [Note **40*R*** → OAT with (*S*)-configuration]
40*S*	Phe	(2*S*)-2-hydroxy-2-phenylethyl GSL (Glucobarbarin)	Kjær and Gmelin (1957b)/ *Barbarea vulgaris*	OrRep: isol + MYR, OAT (UV, compared with chiral synth. ref.); GSL: MS (Bennett et al., 2004; Bianco et al., 2014), NMR (Agerbirk et al., 2001a; Ibrahim et al., 2018, Fig. 14); dGSL: UV, MS, MS2, MS3, NMR (Huang et al., 1994; Agerbirk et al., 2001a, 2015a; Olsen et al., 2016; Ibrahim et al., 2018); NIT: MS (Spencer and Daxenbichler, 1980); OAT: UV, IR, MS, NMR (Radulović et al., 2017) [Note **40*S*** → OAT with (*R*)-configuration]
No. "41" listed in Fahey et al. (2001) ("2-hydroxypropyl GSL") was discontinued due to insufficient evidence (Agerbirk and Olsen, 2012): Claimed ITC unexpected; an OAT would be expected.				
[42]	Met	3-Hydroxypropyl GSL	Daxenbichler et al. (1980)/*Erysimum hieracifolium* = *E. odoratum* and *Malcolmia maritima*	OrRep: ITC (MS, NMR), TMSi of ITC (MS) [Note: There is molecular genetic evidence (Kliebenstein et al., 2001a, 2007) for intermediacy of this GSL in the biosynthesis of **10** from **73**, both of which are conclusively identified. Hence, although from an analytical chemistry point of view, **[42]** still needs verification, the combined evidence is strong.]

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No	Class[a]	Semisystematic name(s) (Trivial name)[b]	First documented report/species[c]	Original and/or selected later critical evidence[d]
43	Trp	Indol-3-ylmethyl GSL (Glucobrassicin)	Gmelin and Virtanen, 1961/*Brassica oleracea*	GSL: UV, IR, MS, MS2, MS3, NMR (Montaut et al., 2010b; Maldini et al., 2017); dGSL: UV, MS, MS2, NMR (Agerbirk et al., 2001b; Zimmermann et al., 2007; Kusznierewicz et al., 2013; Olsen et al., 2016); TMSi of dGSL: EI-MS, CI-MS (Christensen et al., 1982) NIT: GC-MS compared with auth. ref. (Songsak and Lockwood, 2004)
No. "44" listed in Fahey et al. (2001) ("2-methoxybenzyl GSL") is discontinued as OCH_3 group proper position on phenyl ring was not provided in the cited papers. A later claim (Bennett et al., 2004) was only based on MS, which does not provide critical evidence of this GSL either.				
45	Phe	3-Methoxybenzyl GSL (Glucolimnanthin)	Ettlinger and Lundeen (1956b)/*Limnanthes douglasii*	GSL: MS, NMR (Ibrahim et al., 2018); dGSL: UV, MS, NMR (De Nicola et al., 2012; Ibrahim et al., 2018); ITC: MS (Vaughn and Berhow, 2005; De Nicola et al., 2012); NIT: MS (Vaughn and Berhow, 2005)
46	Phe/Tyr	4-Methoxybenzyl GSL (Glucoaubrietin)	Kjær et al. (1956a)/*Aubrieta deltoidea*	GSL: MS (Bennett et al., 2004); dGSL: UV, MS, MS2, NMR (Agerbirk et al., 2008; De Nicola et al., 2012; Olsen et al., 2016); ITC: MS (De Nicola et al., 2012)
47	Trp	1-Methoxyindol-3-ylmethyl GSL, *N*-Methoxyindol-3-ylmethyl GSL (Neoglucobrassicin)	Gmelin and Virtanen (1962)/*Brassica napus*	GSL: UV, IR, MS, NMR (Agerbirk et al., 1998; Montaut et al., 2010b); dGSL: MS, MS2, NMR (Zimmermann et al., 2007; Olsen et al., 2016; Pfalz et al., 2016, Fig. 11B); TMSi of dGSL: EI-MS, CI-MS (Christensen et al., 1982)
48	Trp	4-Methoxyindol-3-ylmethyl GSL (4-Methoxyglucobrassicin)	Truscott et al. (1982b)/*Brassica oleracea*	OrRep: TMSi of dGSL (EI-MS); GSL: UV, MS, NMR (Montaut et al., 2010b); dGSL: UV, MS, MS2, NMR (Agerbirk et al., 2001b; Zimmermann et al., 2007)
[49]	?	2-(4-Methoxyphenyl)-2,2-dimethylethyl GSL	El-Migirab et al. (1977)/*Pentadiplandra brazzeana*	OrRep: ITC (IR, MS, NMR)
50*R*	Phe/Tyr	(2*R*)-2-Hydroxy-2-(4-methoxyphenyl)ethyl GSL	Kjær and Schuster (1972a)/*Arabis hirsuta*	OrRep: OAT (UV, MS, NMR, OptRot, CD); GSL: MS, NMR (Agerbirk et al., 2010b); NIT: MS (Spencer and Daxenbichler, 1980); OAT: MS (Spencer and Daxenbichler, 1980) [Note: **50*R*** → OAT with (*S*)-configuration (OrRep); comparison of dGSL NMR spectra with both epimers of **139** (Agerbirk et al., 2010b)]
51	Ala	Methyl GSL (Glucocapparin)	Kjær et al. (1955b)/*Cleome spinosa*	GSL: MS, NMR (Sakine et al., 2012); dGSL: MS, MS2 (Olsen et al., 2016); TMSi of dGSL: EI-MS, CI-MS (Christensen et al., 1982)
[52]	Leu	3-Methylbut-3-enyl GSL	Kjær and Wagnières (1965)/*Capparis linearis* = *Cynophalla linearis*	OrRep: ITC (IR, MS, NMR)
No. "53" listed in Fahey et al. (2001) is discontinued, since the citation from Daxenbichler et al. (1991) of "1-Methylbutyl GSL" has no basis in the paper.				
54	Ile	(2*S*)-2-Methylbutyl GSL (Glucojiaputin)	Kjær and Friis (1962)/*Putranjiva roxburghii*	OrRep: TU (NMR, comparison of OptRot to that of the synth. enantiomer); GSL: UV, IR, MS, NMR (Montaut et al., 2010b); dGSL: UV, IR, MS, MS2, NMR (Montaut et al., 2010b; Olsen et al., 2016); ITC: MS (Kjær et al., 1963)
[55]	Leu	3-Methylbutyl GSL	Grob and Matile (1980)/*Armoracia lapathifolia* = *A. rusticana*	ITC: MS compared to lit. spectrum (OrRep, Al-Gendy and Lockwood, 2003) [Note: level of evidence very low]
56	Val	1-Methylethyl GSL, Isopropyl GSL (Glucoputranjivin)	Puntambekar (1950)/*Putranjiva roxburghii*	GSL: UV, IR, MS, NMR (Montaut et al., 2010b); dGSL: MS, MS2, NMR (Agerbirk et al., 2008; Olsen et al., 2016: Fig. 11A); TMSi of dGSL: EI-MS, CI-MS (Christensen et al., 1982); ITC: MS (Kjær et al., 1963)
57*R*	Val	(1*R*)-1-Methyl-2-hydroxyethyl GSL, (1*R*)-1-(Hydroxymethyl)ethyl GSL, (Glucosisymbrin)	Kjær and Christensen (1959)/*Sisymbrium austriacum*	OrRep: OAT (comparison of IR and OptRot to that of the synthetic enantiomer); dGSL: MS, MS2, NMR (Agerbirk et al., 2010a; Olsen et al., 2016; Ibrahim et al., 2018) [Note: **57*R*** → OAT with (*R*)-configuration (OrRep)]

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No	Class[a]	Semisystematic name(s) (Trivial name)[b]	First documented report/species[c]	Original and/or selected later critical evidence[d]
58	Ile	3-Methylpentyl GSL	Agerbirk et al. (2008)/*Erucastrum canariense*	OrRep: dGSL (MS, NMR); GSL: UV, IR, MS, NMR (Montaut et al., 2010b); dGSL: MS, MS2, NMR (Agerbirk et al., 2010a; Olsen et al., 2016: Fig. 12B, Suppl. Fig. S1); [Note: Although listed in Fahey et al. (2001), **58** was not reported in quoted papers. Absolute configuration not resolved; believed to be as for Ile]
[59]	Leu	4-Methylpentyl GSL	Kjær et al. (1978)/ *Raphanus sativus* = *R. raphanistrum*	OrRep: ITC (GC-MS compared with synth. ref); ITC: MS (Blažević and Mastelić, 2009)
No. "60" listed in Fahey et al. (2001) ("2-methyl-2-propenyl GSL") is discontinued. This appears to be an undocumented claim or mistyped abbreviation ("MePren" for a trace level peak) (Daxenbichler et al., 1991). A more recent claim (Yang and Quiros, 2010) is based on HPLC-UV retention time only (!).				
61	Ile	(1*S*)-1-Methylpropyl GSL, *sec*-Butyl GSL (Glucocochlearin)	Gadamer (1899c); Hofmann (1874)/*Cochlearia officinalis*	GSL: MS, NMR (Dauvergne et al., 2006); dGSL: MS, MS2, NMR (Agerbirk et al., 2008; Olsen et al., 2016) [Note: This GSL is produced from the natural amino acid L–Ile and presents at C2 an absolute configuration of type *S*. (Kjær and Hansen, 1957)]
62	Leu	2-Methylpropyl GSL, Isobutyl GSL	Underhill and Kirkland (1972)/*Conringia orientalis*	OrRep: ITC (NMR, IR, compared with synth. ref.); dGSL: MS, MS2, NMR (Olsen et al., 2016) GSL: MS, NMR (Sirinut et al., 2017). [Note: The latter report of **62** as a natural product outside the order Brassicales needs analytical confirmation due to the theoretical risk of contamination at reported conditions involving large-scale isolation only].
63	Met	(R_S, 3*E*)-4-(Methylsulfinyl)but-3-enyl GSL[b)] (Glucoraphenin)	Schmid and Karrer (1948)/*Raphanus sativus* = *R. raphanistrum*	GSL: MS, NMR (Maldini et al., 2017; Ibrahim et al., 2018); dGSL: UV, NMR (Iori et al., 2008); ITC: UV, IR, MS, NMR (Kim et al., 2014); NIT: UV, IR, MS, NMR (Kim et al., 2014) [Note: Based on optical rotatory dispersion studies of corresponding TUs (Klyne et al., 1960) the (*R*)-configuration is assigned to all naturally derived sulphoxide mustard oils and their GSL precursors (Cheung et al., 1965). The 3*E*-configuration can be concluded from vicinal proton-proton coupling given in NMR data for dGSL, where *J*vic = 15.2 Hz (Iori et al., 2008)].
64	Met	(R_S)-4-(Methylsulfinyl)butyl GSL (Glucoraphanin)	Procházka (1959)/*Lepidium draba*	GSL: MS, MS2, NMR (Fréchard et al., 2002; Maldini et al., 2017; Ibrahim et al., 2018); dGSL: UV, MS, MS2, (Zimmermann et al., 2007; Kusznierewicz et al., 2013; Olsen et al., 2016), NMR (Kiddle et al., 2001; Ibrahim et al., 2018); ITC: MS, NMR (Kore et al., 1993; Vaughn and Berhow, 2005); NIT: UV, IR, MS, NMR (Vaughn and Berhow, 2005; Kim et al., 2014) [Note: (*R*)-configuration assigned by Cheung et al. (1965). Vergara et al. (2008) analyzed the epimeric purity of **64** by NMR methods using a chiral lanthanide shift reagent. The absolute configuration of the sulfoxide group has been established by comparing the ^{1}H NMR spectra of the two sulfoximine diastereomers of natural **64**. As isolated from broccoli and *A. thaliana*, **64** is a pure epimer and its sulfoxide group has the (*R*) configuration.]
65	Met	(R_S)-10-(Methylsulfinyl)decyl GSL (Glucocamelinin)	Kjær et al. (1956c)/*Camelina sativa*, *C. microcarpa*, and *C. dentata* = *C. alyssum*	GSL: MS, NMR (Berhow et al., 2013); dGSL: MS, MS2 (Olsen et al., 2016); ITC: MS, OptRot (Yamane et al., 1992; Vaughn and Berhow, 2005); NIT: MS (Spencer and Daxenbichler, 1980) [Note: (*R*)-configuration assigned by Cheung et al., 1965]
66	Met	(R_S)-7-(Methylsulfinyl)heptyl GSL	Gmelin et al. (1970)/*Sibara virginica* = *Planodes virginicum*	OrRep: ITC (MS), TU (PC, IR, MS, OptRot); GSL: NMR (Agerbirk et al., 2014); dGSL: MS, MS2, NMR (Olsen et al., 2016); ITC: MS (Spencer and Daxenbichler, 1980); NIT: MS (Spencer and Daxenbichler, 1980) [Note: (*R*)-configuration based on TU OptRot in OrRep]

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Table 1 (*continued*)

No	Class[a]	Semisystematic name(s) (Trivial name)[b]	First documented report/species[c]	Original and/or selected later critical evidence[d]
67	Met	(R_S)-6-(Methylsulfinyl)hexyl GSL (Glucohesperin)	Christensen and Kjær (1963b)/*Hesperis matronalis*	GSL: UV, IR, MS, MS2, NMR (Montaut et al., 2009, 2018; Maldini et al., 2017); ITC: MS (Spencer and Daxenbichler, 1980; Vaughn and Berhow, 2005); NIT: MS (Spencer and Daxenbichler, 1980) [Note: (*R*)-configuration assigned by Cheung et al., 1965]
68	Met	(R_S)-9-(Methylsulfinyl)nonyl GSL (Glucoarabin)	Kjær and Gmelin (1956c)/*Arabis alpina*	GSL: UV, IR, MS, NMR (Berhow et al., 2013; Montaut et al., 2018); dGSL: MS, MS2, NMR (Yamane et al., 1992); ITC: UV, IR, MS, NMR, OptRot (Yamane et al., 1992); NIT: MS (Spencer and Daxenbichler, 1980) [Note: (*R*)-configuration assigned by Cheung et al., 1965]
69	Met	(R_S)-8-(Methylsulfinyl)octyl GSL (Glucohirsutin)	Kjær and Christensen (1958)/*Arabis hirsuta*	GSL: UV, IR, MS, NMR (Montaut et al., 2018); dGSL: MS, MS2, NMR (Yamane et al., 1992; Olsen et al., 2016); ITC: MS, OptRot (Yamane et al., 1992); NIT: MS (Spencer and Daxenbichler, 1980) [Note: (*R*)-configuration assigned by Cheung et al., 1965]
No. "70" listed in Fahey et al. (2001) is discontinued, since no source was given for the "7-Methylsulfinyl-3-oxoheptyl GSL".				
[71]	Met	(R_S)-8-(Methylsulfinyl)-3-oxooctyl GSL	Kjær and Schuster (1972a)/*Arabis hirsuta*	OrRep: ITC (IR, forming TU with aniline), TU (IR, MS) [Note: (*R*)-configuration inferred from optical rotation of the closely analogous (*R*)-1-(8-methylsulfinyloctyl)-3-phenylthiourea (OrRep)]
72	Met	(R_S)-5-(Methylsulfinyl)pentyl GSL (Glucoalyssin)	Kjær and Gmelin (1956a)/*Alyssum argenteum*; Schultz and Wagner (1956a)/*A. argenteum*	GSL: MS, MS2, NMR (Song et al., 2006; Maldini et al., 2017); dGSL: MS, MS2 (Zimmermann et al., 2007); ITC: MS, NMR (Song et al., 2006; Blažević et al., 2010); NIT: MS (Blažević et al., 2010) [Note: (*R*)-configuration assigned by Cheung et al., 1965]
73	Met	(R_S)-3-(Methylsulfinyl)propyl GSL (Glucoiberin)	Schultz and Gmelin (1954)/*Iberis amara*	GSL: MS, MS2, NMR, X-Ray (Jaki et al., 2002; Maldini et al., 2017; Ibrahim et al., 2018); dGSL: UV, MS, MS2, NMR (Zimmermann et al., 2007; Kusznierewicz et al., 2013; Ibrahim et al., 2018); ITC: MS, NMR (Kore et al., 1993; Vaughn and Berhow, 2005); NIT: MS, NMR (Kore et al., 1993; Vaughn and Berhow, 2005) [Note: (*R*)-configuration assigned by Cheung et al., 1965]
[74]	Met	(R_S)-11-(Methylsulfinyl)undecyl GSL	Kjær and Schuster (1972b)/*Neslia paniculata* subsp. *thracica*	OrRep: TU (OptRot, IR, MS); GSL: MS (Berhow et al., 2013); ITC: MS (Spencer and Daxenbichler, 1980); NIT: MS (Spencer and Daxenbichler, 1980) [Note: (*R*)-configuration assigned by OrRep based on OptRot compared to lower homolog]]
No. "75" ("4-(Methylsulfonyl)but-3-enyl GSL") listed in Fahey et al. (2001) is discontinued, since an *m/z* value for the ITC (Cole, 1980) is not sufficient evidence for existence of a natural GSL.				
[76]	Met	4-(Methylsulfonyl)butyl GSL (Glucoerysolin)	Schneider and Kaufmann (1912)/*Erysimum perowskianum*	GSL: MS (Bennett et al., 2004); ITC: MS (Vaughn and Berhow, 2005); NIT: MS (Vaughn and Berhow, 2005)
77	Met	10-(Methylsulfonyl)decyl GSL	Daxenbichler et al. (1991)/*Arabis turrita*	dGSL: MS, NMR (Yamane et al., 1992; Agerbirk et al., 2008); ITC: UV, IR, MS, NMR (Yamane et al., 1992)
[78]	Met	6-(Methylsulfonyl)hexyl GSL	Rodman and Chew (1980)/*Arabis drummondii* = *Boechera stricta*	GSL: MS (Bennett et al., 2004); ITC: MS (Emam and El-Moaty, 2009)
79	Met	9-(Methylsulfonyl)nonyl GSL	Daxenbichler et al. (1991)/*Arabis turrita*, *Capsella bursa-pastoris*, *Heliophila amplexicaulis*, *Rorippa dubia*, *Sinapis arvensis*	GSL: UV, IR, MS, NMR (Fabre et al., 1997); dGSL: MS, MS2, NMR (Yamane et al., 1992; Agerbirk et al., 2008; Olsen et al., 2016); ITC: UV, IR, MS, NMR (Yamane et al., 1992)
80	Met	8-(Methylsulfonyl)octyl GSL	Daxenbichler et al. (1991)/*Arabis turrita*, *Heliophila amplexicaulis*, *Rorippa dubia*, *Sinapis arvensis*	GSL: UV, IR, MS, NMR (Fabre et al., 1997); dGSL: MS, MS2, NMR (Yamane et al., 1992; Agerbirk et al., 2008; Olsen et al., 2016); ITC: UV, IR, MS, NMR (Yamane et al., 1992)
No. "81" ("5-(Methylsulfonyl)pentyl GSL") listed in Fahey et al. (2001) is discontinued. It referred only to a TU derivative observed in PC by Rodman (1976) and "tentatively" suggested to be due to the mentioned GSL. However, PC migration is far from being sufficient evidence for a new GSL.				

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Table 1 (*continued*)

No	Class[a]	Semisystematic name(s) (Trivial name)[b]	First documented report/species[c]	Original and/or selected later critical evidence[d]
82	Met	3-(Methylsulfonyl)propyl GSL (Glucocheirolin)	Schneider (1910)/*Cheiranthus cheiri* = *Erysimum* × *cheiri*	GSL: MS (Bojesen and Larsen, 1991); dGSL: NMR (Huang et al., 1993); ITC: MS (Vaughn and Berhow, 2005; Al-Gendy et al., 2010); NIT: MS (Vaughn and Berhow, 2005)
83	Met	(3*E*)-4-(Methylsulfanyl)but-3-enyl GSL (No trivial name proposed by discoverers of ITC or GSL, "dehydroglucoerucin" is a recommendable derivative name.	Friis and Kjær, 1966/*Raphanus sativus* = *R. raphanistrum*	OrRep: ITC (PC, EC, UV, IR, MS, NMR); GSL: IR, MS, MS2, NMR (Maldini et al., 2017; Visentin et al., 1992; Ibrahim et al., 2018); dGSL: NMR (Ibrahim et al., 2018); ITC: MS (Blažević and Mastelić, 2009); NIT: MS (Blažević and Mastelić, 2009) [Note: Both isomers of ITC (*E*, *Z*) were identified in 4:1 ratio. (OrRep). The double bond configuration was chosen as 3*E* from the NMR coupling constant H3/H4 = 15.0 Hz (Visentin et al., 1992).]
84	Met	4-(Methylsulfanyl)butyl GSL, 4-(Methylthio)butyl GSL (Glucoerucin)	Kjær and Gmelin, 1955/*Eruca sativa* = *E. vesicaria*	GSL: UV, IR, MS, MS2, NMR (Maldini et al., 2017; Ibrahim et al., 2018; Montaut et al., 2018); dGSL: UV, MS, MS2, NMR (Agerbirk et al., 2008; Kusznierewicz et al., 2013; Olsen et al., 2016; Ibrahim et al., 2018); ITC: MS (Blažević and Mastelić, 2009); NIT: MS (Spencer and Daxenbichler, 1980)
[85]	Met	10-(Methylsulfanyl)decyl GSL, 10-(Methylthio)decyl GSL	Daxenbichler et al., 1991/*Arabis amplexicaulis*	OrRep: ITC (MS); NIT: MS (Spencer and Daxenbichler, 1980) (source plant not specified)
No. "86″ listed in Fahey et al. (2001) ("2-(Methylthio)ethyl GSL") was discontinued due to insufficient evidence (Agerbirk and Olsen, 2012). A later claim (Kliebenstein et al., 2001b) was based on an observed minor peak in HPLC-UV of dGSLs and general reference to unpublished results).				
87	Met	7-(Methylsulfanyl)heptyl GSL, 7-(Methylthio)heptyl GSL	Kjær and Schuster (1972a)/*Arabis hirsuta*	OrRep: ITC (MS of impure fractions); GSL: UV, IR, MS, NMR (Montaut et al., 2009, 2018)
88	Met	6-(Methylsulfanyl)hexyl GSL, 6-(Methylthio)hexyl GSL (Glucolesquerellin)	Daxenbichler et al. (1961)/*Lesquerella lasiocarpa* = *Paysonia lasiocarpa*	GSL: UV, IR, MS, NMR (Montaut et al., 2009, 2018); ITC: MS (Vaughn and Berhow, 2005); NIT: MS (Spencer and Daxenbichler, 1980)
[89]	Met	9-(Methylsulfanyl)nonyl GSL, 9-(Methylthio)nonyl GSL	Hasapis et al. (1981)/*Arabis purpurea*	OrRep: ITC (MS); GSL: MS (Bennett et al., 2004); NIT: MS (Spencer and Daxenbichler, 1980) (source plant not specified) [Note: HPLC-MS of dGSL compared with lower homologs (Olsen et al., 2016)]
No. "90" and "91" listed in Fahey et al. (2001) are discontinued since no sources for the "7-methylsulfanyl-3-oxoheptyl-" and "6-methylsulfanyl-3-oxohexyl-" GSLs were given.				
92	Met	8-(Methylsulfanyl)octyl GSL, 8-(Methylthio)octyl GSL	Kjær and Schuster (1972a)/*Arabis hirsuta*	OrRep: ITC and TU (IR and MS compared to auth. ref.); GSL: MS (Bennett et al., 2004); dGSL: MS, MS2, NMR (Olsen et al., 2016); NIT: MS (Spencer and Daxenbichler, 1980)
[93]	Met	8-(Methylsulfanyl)-3-oxooctyl GSL, 8-(Methylthio)-3-oxooctyl GSL	Kjær and Schuster (1972a)/*Arabis hirsuta*	OrRep: ITC (IR, MS, forming TU with NH_3)
94	Met	5-(Methylsulfanyl)pentyl GSL, 5-(Methylthio)pentyl GSL (Glucoberteroin)	Kjær et al. (1955c)/*Berteroa incana*	GSL: UV, IR, MS, NMR (Montaut et al., 2009, 2018); dGSL: UV, MS, NMR (De Nicola et al., 2011); ITC: MS (Blažević et al., 2010); NIT: MS (Blažević et al., 2010)
95	Met	3-(Methylsulfanyl)propyl GSL, 3-(Methylthio)propyl GSL (Glucoibervirin)	Kjær et al. (1955a)/*Iberis sempervirens*	OrRep: TU (PC compared with synth. ref.); GSL: MS, NMR (Chidewe et al., 2017); ITC: MS (Vaughn and Berhow, 2005; Blažević and Mastelić, 2009); NIT: MS (Vaughn and Berhow, 2005)
[96]	?	4-Oxoheptyl GSL (Glucocapangulin)	Kjær et al. (1960)/*Capparis angulata* = *Colicodendron scabridum*	OrRep: Wet chemistry investigations using IR spectroscopy for C═O confirmation, 5-oxooctanoic acid
[97]	?	5-Oxoheptyl GSL (Gluconorcappasalin)	Kjær and Thomsen (1963a)/*Capparis salicifolia*	OrRep: isol + MYR (SO_4^{2-}, Glc), ITC (IR, MS), TU (UV)
[98]	?	5-Oxooctyl GSL (Glucocappasalin)	Kjær and Thomsen (1962b)/*Capparis salicifolia*	OrRep: Ac of GSL (GSL isol., acetylation, UV, IR, elem. comp.), Ac of dGSL (UV, IR, partial 60 MHz NMR), 6-oxononanoic acid methyl ester (MS) [Note: from an analytical chemistry point of view still needs verification, although the evidence is strong]
No. "99" listed in Fahey et al. (2001) ("4-oxopentyl GSL") was discontinued due to lack of any report (Agerbirk and Olsen, 2012). A later claim (Al-Gendy et al., 2010) is not sufficient evidence.				

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Table 1 (*continued*)

No	Class[a]	Semisystematic name(s) (Trivial name)[b]	First documented report/species[c]	Original and/or selected later critical evidence[d]
No. "100" listed in Fahey et al. (2001) ("pent-1-enyl GSL") is discontinued due to lack of any report. It may have been a misreading of the abbreviation Pten (= **101**) in Daxenbichler et al. (1991).				
101	Met	Pent-4-enyl GSL (Glucobrassicanapin)	Kjær and Jensen (1956)/*Brassica napus*	OrRep: TU (IR, compared with synth. ref.); GSL: MS, MS2, MS3 (Millán et al., 2009); dGSL: MS, MS2, NMR (Zimmermann et al., 2007; Olsen et al., 2016); TMSi of dGSL: EI-MS, CI-MS (Christensen et al., 1982); ITC: MS (Blažević et al., 2010); NIT: MS (Blažević et al., 2010)
[102]	?	*n*-Pentyl GSL	Kjær et al. (1978)/*Raphanus sativus* = *R. raphanistrum*	OrRep: ITC (MS compared to auth. ref.); ITC: MS compared to auth. lit. spectrum (Grob and Matile, 1980) [Note: various later claims from HPLC-MS (e.g. Mithen et al., 2010) do not present evidence for *n*-pentyl GSL due to possible isomers]
[103]	?	Phenyl GSL	Kojima et al. (1973)/*Brassica juncea* and *Armoracia rusticana*	ITC: GC-MS compared to auth. ref. (OrRep, Al-Gendy and Lockwood, 2003) (Section 5.2.4.)
[104]	Phe	4-Phenylbutyl GSL	Grob and Matile (1980) *Armoracia lapatifolia* = *A. rusticana*	OrRep: ITC (MS); ITC: GC-MS compared with synth. ref. (Dekić et al., 2017)
105	Phe	2-Phenylethyl GSL, Phenethyl GSL (Gluconasturtiin)	Gadamer (1899d)/*Nasturtium officinale* and *Barbarea praecox* = *B. verna*	GSL: NMR (Ibrahim et al., 2018); dGSL: UV, MS, MS2, NMR (Zimmermann et al., 2007; Agerbirk et al., 2008; Kusznierewicz et al., 2013; Ibrahim et al., 2018); TMSi of dGSL: EI-MS, CI-MS (Christensen et al., 1982); ITC: MS (Blažević and Mastelić, 2009); NIT: MS (Blažević and Mastelić, 2009)
[106]	Phe	3-Phenylpropyl GSL	Grob and Matile (1980) *Armoracia lapatifolia* = *A. rusticana*	OrRep: ITC (MS); ITC: GC-MS compared with synth. ref. (Dekić et al., 2017)
107	Met	Prop-2-enyl GSL Allyl GSL (Sinigrin)	Gadamer (1897b)/*Brassica nigra*	GSL: MS (Bennett et al., 2004.), NMR (Ibrahim et al., 2018); X-Ray (Waser and Watson, 1963; Marsh and Waser, 1970); dGSL: UV, MS, MS2, NMR (Kusznierewicz et al., 2013; Olsen et al., 2016; Ibrahim et al., 2018); TMSi of dGSL: EI-MS, CI-MS (Christensen et al., 1982)
No. "108" listed in Fahey et al. (2001) ("*n*-propyl GSL") is discontinued as it was not reported in any plant. This GSL was included by Fahey et al. (2001) solely based on Fenwick et al. (1983a, 1983b), who mistyped "propyl" GSL instead of "isopropyl" GSL, reported from *Capparis* spp. (Kjær and Thomsen, 1963b).				
109	Phe	2-(*α*-L-Rhamnopyranosyloxy)benzyl GSL	Olsen and Sørensen (1979)/*Reseda odorata*	OrRep: GSL (UV, NMR), TU (UV, NMR); GSL: MS (Bennett et al., 2004); dGSL: MS2 (Agerbirk et al., 2018)
110	Phe/Tyr	4-(*α*-L-Rhamnopyranosyloxy)benzyl GSL (Glucomoringin)	Badgett (1964)/*Moringa oleifera*	OrRep: ITC (reactions, peracetyl derivative, NMR); GSL: compared with synth. ref. (Gueyrard et al., 2000), MS, MS2 (Maldini et al., 2014), NMR (Ibrahim et al., 2018); dGSL: MS, NMR (de Graaf et al., 2015; Ibrahim et al., 2018); ITC: UV, IR, MS, MS2, NMR, (Kjær et al., 1979; de Graaf et al., 2015; Müller et al., 2015); NIT: NMR (Faizi et al., 1994); TU: UV, MS, MS2, NMR (Agerbirk et al., 2015b)
111	Met	(R_S, 3*E*)-6′-Sinapoyl-4-(methylsulfinyl)but-3-enyl GSL (6′-Sinapoylglucoraphenin)	Linscheid et al. (1980)/*Raphanus sativus* = *R. raphanistrum*	OrRep: GSL (MS, NMR); dGSL: UV, IR, MS, NMR (Kim et al., 2014)
112	Trp	1-Sulfoindol-3-ylmethyl GSL, *N*-sulfoindol-3-ylmethyl GSL, (Glucobrassicin-*N*-sulfonate, *N*-sulfoglucobrassicin)	Elliott and Stowe (1970)/*Isatis tinctoria*	OrRep: GSL (UV, IR, NMR); GSL: MS (Bennett et al., 2004) [Note: The sulfo group is attached directly on N atom of indole ring via N–S bond, not N–O–S bond as wrongly drawn in Fahey et al., 2001]
[113]	?	4,5,6,7-Tetrahydroxydecyl GSL	Gaind et al. (1975)/*Capparis grandis*	OrRep: isol. + MYR, Glc, ITC (UV, IR, NMR) [Note: from an analytical chemistry point of view still needs verification, although the evidence is strong. Absolute configuration is not resolved (OrRep)]
114	Phe/Tyr	3,4,5-Trimethoxybenzyl GSL	Kjær and Wagnières (1971)/*Lepidium sordidum*	GSL: MS, MS2 (Maldini et al., 2017); dGSL: UV, MS, MS2, NMR (Pagnotta et al., 2017); ITC: MS (Radulović et al., 2008; Pagnotta et al., 2017); NIT: MS (Pagnotta et al., 2017)
No. "115" and "116" listed in Fahey et al. (2001) ("iso"-heptyl GSL; "iso"-hexyl GSL) were discontinued (Agerbirk and Olsen, 2012), since such names do not correspond to clearly defined structures.				

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Table 1 (*continued*)

No	Class[a]	Semisystematic name(s) (Trivial name)[b]	First documented report/species[c]	Original and/or selected later critical evidence[d]
117	Met	5-(Benzoyloxy)pentyl GSL	Hogge et al. (1988)/*Arabidopsis thaliana*	OrRep: ITC (GC-MS of minor peak); dGSL: MS, MS2, NMR, esterase-products (Reichelt et al., 2002)
[118]	Met	6-(Benzoyloxy)hexyl GSL	Hogge et al. (1988)/*Arabidopsis thaliana*	OrRep: ITC (GC-MS of minor peak, MS and t_R compared with known lower homolog) [Note: level of evidence very low]
No. "119" and "120" listed in Fahey et al. (2001) (apiosyl and acyl derivatives of **[14]**) were discontinued (Agerbirk and Olsen, 2012), since Fahey et al. (2001) concluded that these claims (Larsen et al., 1992) were non-documented. A later report of apparent GSLs with corresponding nominal masses in the species (*Hesperis matronalis*) is discussed in Section 2.7.).				
121*R*	Met	2′,3′-Dihydro-2′-oxoindol-3′-ylacetate ester at 2-OH of (*R*)-2-hydroxybut-3-enyl GSL (Glucoisatisin)	Fréchard et al. (2001)/*Isatis tinctoria*	OrRep: GSL (UV, MS, NMR); GSL: MS, (Mohn and Hamburger, 2008) [Note: The stereochemistry was deduced from NMR spectra, and supported by the interpretation of the NMR spectrum of a 4:1 mixture of progoitrin (*R*) and epiprogoitrin (*S*). However, it was not possible to unambiguously determine the configuration of carbon C3′ given rotational freedom in the side chain and the lack of significant NOEs. (OrRep)]
121*S*	Met	2′,3′-Dihydro-2′-oxoindol-3′-ylacetate ester at 2-OH of (*S*)-2-hydroxybut-3-enyl GSL (Epiglucoisatisin)	Fréchard et al. (2001)/*Isatis tinctoria*	OrRep: GSL (UV, MS, NMR); GSL: MS (Mohn and Hamburger, 2008) [Note: the same as for **121*R***]
122*R*	Met	2′,3′-Dihydro-3′-hydroxy-2′-oxoindol-3′-ylacetate ester at 2-OH of (*R*)-2-hydroxybut-3-enyl GSL ((*R*)-3′-Hydroxyglucoisatisin)	Fréchard et al. (2001)/*Isatis tinctoria*	OrRep: GSL (UV, MS, NMR) [Note: the same as for **121*R***]
122*S*	Met	2′,3′-Dihydro-3′-hydroxy-2′-oxoindol-3′-ylacetate ester at 2-OH of (*S*)-2-hydroxybut-3-enyl GSL ((*S*)-3′-Hydroxyepiglucoisatisin)	Fréchard et al. (2001)/*Isatis tinctoria*	OrRep: GSL (UV, MS, NMR) [Note: the same as for **121*R***]
[123*S*]	Met	(2*S*)-2-Benzoyloxybut-3-enyl GSL (2-*O*-Benzoylepiprogoitrin)	Reichelt et al. (2002)/*Arabidopsis thaliana*	OrRep: dGSL (MS, MS2, esterase-products) [Note: Esterase-treatment of the dGSL gave d**24*S***, demonstrating the 2*S* configuration]
[124]	Met	3-Sinapoyloxypropyl GSL	Kliebenstein et al. (2007)/*Arabidopsis thaliana*	OrRep: dGSL (MS3, NMR claimed not reported)
125	Met	6′-Benzoyl-4-benzoyloxybutyl GSL	Reichelt et al. (2002)/*Arabidopsis thaliana*	OrRep: dGSL (UV, MS, MS2, NMR, esterase-products)
126	Met	6′-Benzoyl-4-(methylsulfinyl)butyl GSL (6′-Benzoylglucoraphanin)	Reichelt et al. (2002)/*Arabidopsis thaliana*	OrRep: dGSL (UV, MS, MS2, NMR, esterase-products)
127	Met	6′-Benzoyl-4-(methylsulfanyl)butyl GSL (6′-Benzoylglucoerucin)	Reichelt et al. (2002)/*Arabidopsis thaliana*	OrRep: dGSL (UV, MS, MS2, NMR, esterase-products)
No. "128" listed in Agerbirk and Olsen (2012) was discontinued by Agerbirk and Olsen (2013), since the NMR spectral evidence of the relevant report of "6′-(*p*-coumaroyl) glucoraphanin" (Survay et al., 2010) exhibited inexplicable features and since a later, related report by the same authors was retracted.				
129	Phe	6′-Isoferuloyl-2-phenylethyl GSL, 6′-Isoferuloylphenethyl GSL (6′-Isoferuloylgluconasturtiin)	Agerbirk and Olsen (2011)/*Barbarea vulgaris*	OrRep: GSL (*m/z*), dGSL (UV, MS2, NMR, esterase-products); GSL: MS (Bianco et al., 2014)
130	Trp	6′-Isoferuloylindol-3-ylmethyl GSL (6′-Isoferuloylglucobrassicin)	Agerbirk and Olsen (2011)/*Barbarea vulgaris*	OrRep: GSL (*m/z*), dGSL (UV, MS2, NMR, esterase-products); GSL: MS (Bianco et al., 2014)
131*R*	Phe	6′-Isoferuloyl-(2*R*)-2-hydroxy-2-phenylethyl GSL (6′-Isoferuloylepiglucobarbarin)	Agerbirk and Olsen (2011)/*Barbarea vulgaris*	OrRep: GSL (MS, NMR), dGSL (UV, MS2, NMR, esterase-products)
131*S*	Phe	6′-Isoferuloyl-(2*S*)-2-hydroxy-2-phenylethyl GSL (6′-Isoferuloylglucobarbarin)	Agerbirk and Olsen (2011)/*Barbarea vulgaris*	OrRep: GSL (MS, NMR), dGSL (UV, MS2, NMR, esterase-products); GSL: MS (Bianco et al., 2014)
132*R*	Phe/Tyr	6′-Isoferuloyl-(*R*)-2-hydroxy-2-(4-hydroxyphenyl)ethyl GSL	Agerbirk and Olsen (2011)/*Barbarea vulgaris*	OrRep: GSL (*m/z*), dGSL (UV, MS2, NMR, esterase-products: Fig. 11C)
133	Met	4-Mercaptobutyl GSL [Note: "glucosativin" and "sativin" for the ITC are widely used as trivial names but problematic: not suggested by original discoverer, derived from scientific plant name now abandoned, and "sativin" already in use for an isoflavan and a protein]	Bennett et al. (2002)/*Eruca sativa* = *E. vesicaria*	OrRep: GSL (MS, NMR performed on the purified dimer **134**), ITC (MS); GSL: MS, MS2 (Cataldi et al., 2007); 1,3-Thiazepane-2-thione: NMR (Fechner et al., 2018) [Note: It was suggested that disulfide **134** is formed through a non-enzymatic oxidation of **133** during the extraction and purification steps. (OrRep)]
134	Met	"Dimeric 4-mercaptobutyl GSL"	Cerny et al. (1996)/*Eruca sativa* = *E. vesicaria*	OrRep: *bis*(ITC) (IR, MS, NMR); GSL: MS, MS2, NMR (Bennett et al., 2002; Cataldi et al., 2007); dGSL: MS (Bennett et al., 2002); *bis*(ITC): MS (Bennett et al., 2002); ITC-NIT: MS (Bennett et al., 2002); *bis*(NIT): MS (Bennett et al., 2002) [Note: Although in some cases perhaps an extraction artifact, **134** was concluded as natural after critical experiments by Cataldi et al. (2007).]

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No	Class[a]	Semisystematic name(s) (Trivial name)[b]	First documented report/species[c]	Original and/or selected later critical evidence[d]
135	Met	4-(β-D-Glucopyranosyldisulfanyl)butyl GSL, 4-(β-D-Glucopyranosyldithio)-butyl GSL (Diglucothiobeinin)	Kim et al. (2004)/*Eruca sativa* = *E. vesicaria*	OrRep: dGSL (MS, NMR); GSL: MS (Lelario et al., 2012) [Note: trivial name suggested by Kim et al., 2007]
136	Met	(*R*)-4-(Cystein-*S*-yl)butyl GSL (Glucorucolamine)	Kim et al. (2007)/*Eruca sativa* = *E. vesicaria*	OrRep: dGSL (MS, NMR) [Note: only described from plants grown at specific hydroponic conditions and never confirmed by any later author, so it remains possible that it only occurs at "artificial conditions" as further discussed in Section 2.6.]
137	Met	2-Methylsulfinylethyl GSL	Reichelt et al. (2002)/*Arabidopsis thaliana*	OrRep: dGSL (UV, MS, MS2, NMR)
138	Trp	1,4-Dimethoxyindol-3-ylmethyl GSL (1,4-Dimethoxyglucobrassicin)	Agerbirk et al. (2001b)/*Barbarea vulgaris*	OrRep: dGSL (UV, MS, NMR). Graph of UV spectrum in Agerbirk et al. (2010a). Ion trap MS2 in Olsen et al. (2016).
139*R*	Phe/Tyr	(2*R*)-2-Hydroxy-2-(4-hydroxyphenyl)ethyl GSL (*p*-Hydroxyepiglucobarbarin)	Agerbirk et al. (2001a)/*Barbarea vulgaris*	OrRep: dGSL (UV, MS, NMR); GSL: MS, NMR (Agerbirk et al., 2010a); Thiazolidin-2-one: UV, IR, MS, NMR (Agerbirk and Olsen, 2015); NIT: MS, MS2, NMR (Agerbirk et al., 2010b, 2018)
139*S*	Phe/Tyr	(2*S*)-2-Hydroxy-2-(4-hydroxyphenyl)ethyl GSL (*p*-Hydroxyglucobarbarin)	Agerbirk et al. (2001a)/*Barbarea orthoceras*	OrRep: dGSL (UV, MS, NMR)
140	Phe/Tyr	2-(4-Hydroxyphenyl)ethyl GSL *p*-Hydroxyphenethyl GSL (Homosinalbin)	Bennett et al. (2004) and[c] Agerbirk et al. (2008)/*Arabis hirsuta*	OrRep: GSL (MS, t_R); GSL: MS, NMR (Agerbirk et al., 2010b); dGSL: UV, MS, NMR (Agerbirk et al., 2008); NIT: UV, MS, NMR (Agerbirk et al., 2010b, 2018)
141	Ile	3-(Hydroxymethyl)pentyl GSL	Agerbirk et al. (2010a)/*Cardamine pratensis*	OrRep: dGSL (MS, NMR); GSL: NMR (Fig. 13, Section 7.2, Suppl. Fig. S2) [Note: Absolute configuration not resolved; believed to be as for Ile]
142*R*	Phe	(2*R*)-2-Hydroxy-2-(3-hydroxyphenyl)ethyl GSL (*m*-Hydroxyepiglucobarbarin)	Agerbirk and Olsen (2012)/*Barbarea vulgaris*	OrRep: GSL (NMR, MS, UV), dGSL (NMR, MS, UV); OAT: UV, MS, NMR (OrRep, Agerbirk and Olsen, 2015) [Note: The epimer "**142*S***" tentatively suggested by MS (Agerbirk et al., 2015a)]
143	Phe/Tyr	2-(4-Methoxyphenyl)ethyl GSL *p*-Methoxyphenethyl GSL	Bennett et al. (2004) and[c] Agerbirk et al. (2008)/*Arabis soyeri* subsp. *subcoriacea*	OrRep: GSL (MS, t_R); GSL: NMR (Agerbirk et al., 2010b); dGSL: MS, NMR (Agerbirk et al., 2008) [Note: All three reports used the same seed batch under varying botanical names, see Agerbirk et al. (2010b) (Sec. 2.1.)]
[144] Art	Se-Met	3-(Methylseleno)propyl GSL OrRep proposed the name ("Glucoselenoiberverin") but comparison with **95** suggest an i/e typing mistake, making "Glucoselenoibervirin" preferable	Matich et al. (2012)/produced in *Brassica* spp. fertilised with sodium selenate	OrRep: ITC (MS), NIT (GC-MS compared with synth. ref.), GSL (HPLC-MS compared with natural S-analog) [Note on **[144]**, **[145]**, **[146]**: These so far partially characterized GSLs of plant origin only reported from plants exposed to unusually high levels of selenate. Although analytical evidence is strong, NMR of the GSL or dGSL would be preferable. If these GSLs can be confirmed, it seems likely that they would also exist (at lower levels) in plants grown at lower, more usual levels of Se, but this assumption also needs verification.
[145] Art	Se-Met	4-(Methylseleno)butyl GSL (Glucoselenoerucin)	Matich et al. (2012)/as for **144**	OrRep: ITC (GC-MS compared with synth. ref.), NIT (GC-MS compared with synth. ref.), GSL (HPLC-MS compared with natural S-analog) [See note at **[144]**]
[146] Art	Se-Met	5-(Methylseleno)pentyl GSL (Glucoselenoberteroin)	Matich et al. (2012)/as for **144**	OrRep: ITC (MS), GSL (HPLC-MS compared with natural S-analog) [See note at **[144]**]
[147]	Trp	4-Methoxyindol-3-yl GSL (Glucorapassicin A)	Pedras et al. (2007)/*Brassica rapa*	OrRep: ITC (Isolated as the phytoalexin rapalexin A (3-isothiocyanato-4-methoxy-1*H*-indole), synth., UV, IR, MS, NMR); GSL: Unstable, deduced hypothetical GSL never isolated. Existence and instability deduced from existence of ITC, biosynthetic exp. using isotope-labeled precursors and attempted chemical synthesis. The dGSL has also been synthesized, but not isolated from nature, and is hence not critical evidence for existence of **[147]** in nature (Pedras and Yaya, 2013; Pedras et al., 2016)

(*continued on next page*)

Table 1 (*continued*)

No	Class[a]	Semisystematic name(s) (Trivial name)[b]	First documented report/species[c]	Original and/or selected later critical evidence[d]
148	Phe	2-(3-Hydroxyphenyl)ethyl GSL *m*-Hydroxyphenethyl GSL	Agerbirk et al. (2015a)/*Barbarea vulgaris*	OrRep: dGSL (UV, MS, NMR)
149	Ile	2-Hydroxy-3-methylpentyl GSL	Olsen et al. (2016)/*Cardamine pratensis*	OrRep: dGSL (MS, NMR: Fig. 12B), High res. MS (Agerbirk et al., 2010a) [Note: Absolute configuration not resolved; believed to be as for Ile]
No. "150" (2-Hydroxy-8-(methylsulfinyl)octyl GSL) listed in Olsen et al. (2016)is discontinued since the evidence (MS2 of dGSL and comparison of t_R with **d69**) is too preliminary.				
151	Phe/Tyr	4-Hydroxy-3-methoxybenzyl GSL (3-Methoxysinalbin)	Pagnotta et al. (2017)/*Lepidium densiflorum*	OrRep: dGSL (UV, MS, MS2, NMR), NIT and carboxylic acid tentative product (MS); ITC: MS (Montaut et al., 2015)
152	Phe/Tyr	4-Hydroxy-3,5-dimethoxybenzyl GSL (3,5-dimethoxysinalbin)	Pagnotta et al. (2017)/*Lepidium densiflorum*	OrRep: dGSL (UV, MS, MS2, NMR), NIT (GC-MS compared with auth. ref.), aldehyde and alcohol (tentative MS)
153	Phe/Tyr	3-Hydroxy-4-methoxybenzyl GSL (Glucobretschneiderin)	Montaut et al. (2015)/*Bretschneidera sinensis*	OrRep: GSL (UV, IR, MS, NMR)
[154]	Trp	1-Hydroxyindol-3-ylmethyl GSL *N*-Hydroxyindol-3-ylmethyl GSL *N*-hydroxyglucobrassicin	Pfalz et al. (2016)/*Arabidopsis thaliana* mutants	OrRep: GSL (MS), dGSL (UV, MS2) [Note: Unstable. Additional genetic evidence: Correlated with deletion of the methyl transferase gene *IGMT5* and lack of **47** (OrRep)]
[155]	Glu	3-Carboxypropyl GSL	Radulović et al. (2011)/*Erysimum diffusum*	OrRep: ITC (GC-MS compared with synth. ref. after methylation)
[156]	Phe	5-Phenylpentyl GSL (Glucoarmoracin)	Dekić et al. (2017)/*Armoracia rusticana*	OrRep: ITC (GC-MS compared with synth. ref.)

[a] Known or likely proteinogenic amino acid biosynthetic precursor of the side chain, either concluded from experimental evidence in at least one species, from extrapolation of such evidence, or from side chain skeleton (Section 2.3.). **[147]** is derived from Trp in an atypical way, via a chain-shortening mechanism via an indol-3-ylmethyl GSL and a substituted indolylSer non-proteinogenic amino acid, see text (Sections 1.1.2. and 4.4.).

[b] For GSLs with just one chiral center in the side chain, the designation of the position of the chiral center by a number or an index "S" is optional (e.g. another correct name of **24*R*** is (*R*)-2-Hydroxybut-3-enyl GSL). Listed trivial names are as proposed by original discoverer or a recommendable derivative name constructed from such trivial name. Derivative names consist of a prefix as used in systematic chemical names followed by a trivial name, e.g. 4-methoxyglucobrassicin, dehydroglucoerucin. Later trivial names, typically arising from typing errors etc., are not listed.

[c] As "discoverer" we list the first substantially documented report with source plant identification of either an aglucon product or the GSL/dGSL. The species name first mentioned is the one actually used in the original report. In case this name is not currently accepted according to www.theplantlist.org, the currently accepted name is also given. Botanical authorities are left out for space considerations. For the currently accepted names, they are as follows:

Akaniaceae: *Bretschneidera sinensis* Hemsl.

Brassicaceae: *Alyssum argenteum* All.; *Alyssum argenteum* Vitman (unresolved); *Arabidopsis thaliana* (L.) Heynh.; *Arabis alpina* L.; *Arabis amplexicaulis* Edgew.; *Arabis hirsuta* (L.) Scop.; *Arabis purpurea* Sm.; *Arabis soyeri* Reut & Huet subsp. *subcoriacea* (Gren.) Breistr.; *Arabis turrita* L.; *Armoracia rusticana* P.Gaertn., B.Mey. & Scherb.; *Aubrieta deltoidea* (L.) DC.; *Barbarea orthoceras* Ledeb.; *Barbarea verna* (Mill.) Asch.; *Barbarea vulgaris* R.Br.; *Berteroa incana* (L.) DC.; *Brassica juncea* (L.) Czern.; *Brassica napus* L.; *Brassica nigra* (L.) K. Koch; *Brassica oleracea* L.; *Brassica rapa* L.; *Boechera stricta* (Graham) Al-Shehbaz; *Camelina alyssum* (Mill.) Thell.; *Camelina microcarpa* Andrz. ex DC.; *Camelina sativa* (L.) Crantz; *Capsella bursa-pastoris* (L.) Medik.; *Cardamine pratensis* L.; *Cochlearia officinalis* L.; *Conringia orientalis* (L.) Dumort.; *Crambe hispanica* subsp. *abyssinica* (Hochst. ex R.E.Fr.) Prina; *Eruca vesicaria* (L.) Cav.; *Erucastrum canariense* Webb & Berthel.; *Erysimum* × *cheiri* (L.) Crantz; *Erysimum diffusum* Ehrh.; *Erysimum odoratum* Ehrh.; *Erysimum perowskianum* Fisch. & C.A. Mey. (unresolved); *Erysimum pulchellum* (Willd.) J. Gay.; *Erysimum rhaeticum* (Schleich. ex Hornem.) DC.; *Eutrema japonicum* (Miq.) Koidz.; *Heliophila amplexicaulis* L.f.; *Heliophila coronopifolia* L.; *Hesperis matronalis* L.; *Iberis amara* L.; *Iberis sempervirens* L.; *Isatis tinctoria* L.; *Lepidium draba* L.; *Lepidium densiflorum* Schrad.; *Lepidium graminifolium* L.; *Lepidium sordidum* A. Gray; *Lepidium virginicum* subsp. *menziesii* (DC.) Thell.; *Malcolmia maritima* (L.) R.Br.; *Moricandia arvensis* (L.) DC.; *Nasturtium officinale* R.Br.; *Neslia paniculata* subsp. *thracica* (Velen.) Bornm.; *Paysonia lasiocarpa* (Hook. ex A. Gray) O'Kane & Al-Shehbaz; *Planodes virginicum* (L.) Greene; *Raphanus raphanistrum* subsp. *sativus* (L.) Domin; *Rorippa dubia* (Pers.) H.Hara; *Sinapis arvensis* L.; *Sisymbrium austriacum* Jacq.

Capparaceae: *Capparis grandis* L.f.; *Capparis salicifolia* Griseb.; *Capparis sikkimensis* subsp. *masaikai* (H.Lév.) M.Jacobs; *Cynophalla flexuosa* (L.) J.Presl; *Cynophalla linearis* (Jacq.) J.Presl; *Colicodendron scabridum* (Kunth) Seem.

Cleomaceae: *Cleome spinosa* Jacq.

Limnanthaceae: *Limnanthes douglasii* R. Br.

Moringaceae: *Moringa oleifera* Lam.; *Moringa peregrina* (Forssk.) Fiori

Pentadiplandraceae: *Pentadiplandra brazzeana* Baill.

Putranjivaceae: *Putranjiva roxburghii* Wall.

Resedaceae: *Reseda odorata* L.; *Reseda phyteuma* L.; *Sesamoides interrupta* (Boreau) G.López

Tovariaceae:*Tovaria pendula* Ruiz & Pav

Tropaeolaceae: *Tropaeolum majus* L.

[d] Only critical evidence for the existence in nature is given, not general spectroscopic data for the corresponding synthetic compound. Level of detail is deliberately not uniform, tending to give relatively more detail in critical or unusual cases. OrRep, Original report. Abbreviations for analytes: GSL, glucosinolate; dGSL, desulfoglucosinolate, ITC, isothiocyanate, NIT, nitrile; OAT, oxazolidine-2-thione, THOT, tetrahydro-1,3-oxazine-2-thione; TU, thiourea-type derivative. Abbreviations for kinds of data or reagents: auth., authentic; CD, circular dichroism; GC-MS, gas chromatography-mass spectrometry; IR, infrared spectroscopy; isol + MYR, isolation of the GSL followed by demonstration of sensitivity to myrosinase and identification of the listed products (in case of glucose abbreviated Glc); MS, mass spectrometry; MYR, myrosinase; *m/z*, *m/z* value only from MS; NMR, NMR spectroscopy; OptRot, Optical Rotation; PC, paper chromatography; ref., reference; synth., synthetic; UV, UV spectroscopy; X-ray, X-ray crystallography, elem. comp., elemental composition analysis.

alternative formation of ITCs has been demonstrated in a lower plant (von Reuss and von König, 2005) and in bacteria (Li et al., 2015; Flórez et al., 2017), the latter of which might occur as endophytes or pathogens in investigated plants. Chemical transformations during GC (Section 5.2.5.) or misidentification in early GC-MS with much lower resolution than in present day instruments, such as the often questioned pioneering paper by Cole (1976) (e.g. Larsen et al., 1983; Olsen et al., 2016), are other potential factors to take into account in these cases.

Enzymatic desulfation of drug metabolites was developed in pharmaceutical sciences and the application to GSL discovery (Thies, 1979;

Table 2
Location of glucosinolates in panels A–I of Fig. 15.

[1]	A	**[25]**	I	**47**	C	**69**	G	**95**	G	**121*S***	I	**139*S***	E
[2]	C	**[26]**	I	**48**	C	**[71]**	H	**[96]**	A	**122*R***	I	**140**	E
[3]	F	**27**	I	**[49]**	A	**72**	G	**[97]**	A	**122*S***	I	**141**	B
4	F	**28**	C	**50*R***	E	**73**	G	**[98]**	A	**[123*S*]**	I	**142*R***	E
5	I	**29*S***	B	**51**	A	**[74]**	G	**101**	I	**[124]**	I	**143**	E
[6]	I	**30**	B	**[52]**	B	**[76]**	G	**[102]**	A	**125**	I	**[144]**	H
[7]	B	**31**	B	**54**	B	**77**	G	**[103]**	A	**126**	H	**[145]**	H
[9]	A	**[32]**	H	**[55]**	B	**[78]**	G	**[104]**	E	**127**	H	**[146]**	H
[10]	I	**[33]**	H	**56**	A	**79**	G	**105**	E	**129**	F	**[147]**	C
11	D	**[34]**	H	**57**	A	**80**	G	**[106]**	E	**130**	C	**148**	E
12	I	**[35]**	H	**58**	B	**82**	G	**107**	I	**131*R***	F	**149**	B
[13]	A	**[36]**	H	**[59]**	B	**83**	H	**109**	F	**131*S***	F	**151**	D
[14]	D	**[37]**	H	**61**	B	**84**	G	**110**	F	**132*R***	F	**152**	D
15	D	**38*S***	I	**62**	B	**[85]**	G	**111**	H	**133**	H	**153**	D
[16]	A	**40*R***	E	**63**	H	**87**	G	**112**	C	**134**	H	**[154]**	C
[20]	A	**40*S***	E	**64**	G	**88**	G	**[113]**	A	**135**	H	**[155]**	A
22	D	**[42]**	I	**65**	G	**[89]**	G	**114**	D	**136**	H	**[156]**	E
23	D	**43**	C	**66**	G	**92**	G	**117**	I	**137**	G		
24*R*	I	**45**	D	**67**	G	**[93]**	H	**[118]**	I	**138**	C		
24*S*	I	**46**	D	**68**	G	**94**	G	**121*R***	I	**139*R***	E		

Sang and Truscott, 1984; Sang et al., 1984) was a major advance. Briefly, the analysis consists of extracting GSLs under conditions that inactivate myrosinase, passing the extract through an anion exchange cartridge to bind all anions, then adding sulfatase enzyme to allow enzymatic hydrolysis of the sulfate ester bonds in GSLs to form desulfoGSLs (dGSLs), and finally eluting the uncharged dGSLs from the cartridge. The technique allows semi-quantitative analysis of the vast majority of GSLs (Section 5.1.4.). Notably, apart from the missing sulfate, the corresponding GSL and dGSL are identical, allowing structure elucidation (for an exception, see Section 5.1.4.). Numerous GSLs have been discovered using this method (e.g. Truscott et al., 1982a, 1982b; Reichelt et al., 2002). Since the binding to an anion exchanger confirms the presence of the sulfate group (or another negatively charged group) in the native molecule, the present reviewers accept identified dGSLs as evidence for the corresponding GSLs. In many cases, the intact GSLs have subsequently been confirmed (Table 1). In one documented case, however, insect metabolites that are not GSLs will actually form dGSLs in the standard dGSL procedure (Opitz et al., 2011). This kind of metabolite (a GSL or dGSL sulfated at the glucose moiety) is so far unknown from GSL-containing plants.

Isolation of intact GSLs from plant material dominated by only one GSL has been optimized to perfection (Thies, 1988; Visentin et al., 1992; Barillari et al., 2005a). Some members of this group of GSLs are commercially available. However, the separation of multiple GSLs from complex samples has been a challenge. Although ion-pairing HPLC of intact GSLs was reported quite early (Helboe et al., 1980), the technique was not adapted commonly, possibly because the use of highly concentrated salt eluents deterred HPLC-managers and because the resulting chromatograms were less satisfactory than dGSL chromatograms and contained non-GSL peaks. More recently, it was discovered (but never theoretically explained, Agerbirk and Olsen, 2012) that introducing weak acids in HPLC eluents allowed a comparable separation (Mellon et al., 2002; Agerbirk et al., 2014). With these techniques (and several others such as differential elution from DEAE-Sephadex ion exchange columns and other columns), isolation of novel intact GSLs from complex samples is reported more frequently now (Fréchard et al., 2001; Agerbirk and Olsen, 2011; Agerbirk and Olsen, 2012; Montaut et al., 2015). Structure elucidation of intact GSLs isolated under mild conditions is the ultimate proof of existence of the GSL in question.

Irrespective of the compound classes at hand - intact GSLs, degradation products or dGSLs - the actual reported structural evidence is obviously critical for the scientific value of the result. Unfortunately, the modern literature contains many unsubstantiated or non-conclusive claims, but the mere claim of a structure in a scientific journal is not scientific evidence. For a scientific report of a structure to be accepted, the present reviewers demand that the standards of dedicated natural product journals are met. This means that extensive spectroscopic characterization should be reported, including at least ^{1}H NMR, MS and satisfactory interpretation. Further spectroscopic evidence, including ^{13}C NMR, MS/MS, elemental composition, comparison with a synthetic specimen etc. is obviously advantageous. Frequently, chemical evidence (e.g. isolation, susceptibility to myrosinase and identification of hydrolysis products including aglucone products, sulfate and glucose) is relevant too. When well carried out, this is strong evidence (e.g. **[1]**, **[9]**, **[10]**) (Table 1).

2.2. NMR of glucosinolates and desulfoglucosinolates

The final confirmation of a GSL structure is usually based on NMR of GSLs or dGSLs. NMR allows unequivocal determination of structures. While the isolation of GSLs can be more demanding than isolation of dGSLs, their NMR analysis is equivalent and will be explained and reviewed together here. The initial explanation will be based on one-dimensional proton NMR (^{1}H NMR), while carbon NMR (^{13}C NMR) and "two-dimensional" (2D) techniques are covered only superficially. Some previous papers have provided elaborated examples of various types of spectra, using the same GSLs (**73** and **45**) throughout (Jaki et al., 2002; Ibrahim et al., 2018). Here, a complementary approach will be used, with focus on the variation of ^{1}H NMR spectral appearance depending on GSL structure, supplemented with one complete set of spectra for one GSL and one dGSL (Supplementary Fig. S1 and Supplementary Fig. S2).

In the early days of NMR, large amounts of sample were required. However, for decades, NMR has been very sensitive allowing good spectra from fractions of a mg; manual collection of trace peaks from a classical analytical HPLC column (e.g. 250 mm × 4.5 mm) has allowed NMR distinction of many known isomers and of many novel GSLs. Such NMR verification is feasible at analytical scale (Agerbirk et al., 2010a, 2014, 2015a; Agerbirk and Olsen, 2011; Olsen et al., 2016). The essential trick is injection of much more than usual analytical amounts, e.g. 50 fold more per injection. Modern HPLC columns have plenty of capacity for such loading. After evaporation of the solvent, neutral fractions with dGSLs can be dissolved directly in NMR solvents, usually D_2O. Acidic HPLC fractions with intact GSLs must be neutralized before evaporation of the solvent, to avoid hydrolysis (Agerbirk et al., 2014).

Usually, we know the presence of a negatively charged sulfate group already from binding to ion exchange columns, HPLC-mobility or loss of sulfate in MS/MS. Susceptibility to myrosinase is another early indication of a GSL, including the presence of the sulfate group. An obvious NMR-indication of a GSL or dGSL is the anomeric proton signal

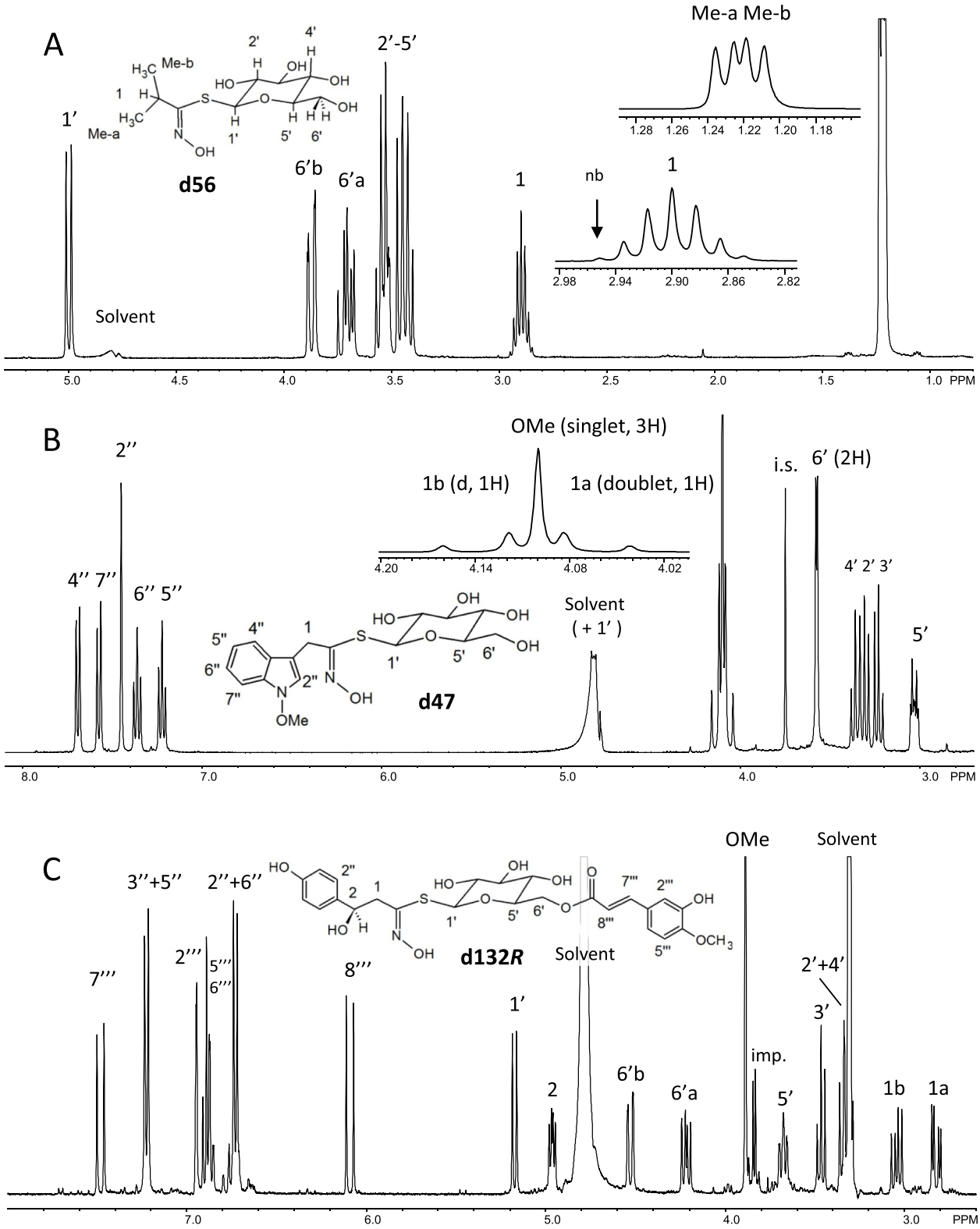


Fig. 11. Representative ^{1}H NMR spectra of desulfoglucosinolates (dGSLs). (A) Isopropyl dGSL (**d56**) in D_2O, (Olsen et al., 2016). (B) *N*-Methoxyindol-3-ylmethyl dGSL (**d47**) in D_2O, internal standard (i.s.) = dioxane (Olsen et al., 2016). (C) 6′-Isoferuloyl-(*R*)-2-hydroxy-2-(4-hydroxyphenyl)ethyl dGSL (**d132*R***) in CD_3OD (Agerbirk and Olsen, 2011).

(usually a doublet) at *ca.* 4.5–5.0 ppm (further downfield than the other carbohydrate protons, due to the simultaneous proximity to the S and O). Next, we notice the signals from the remaining C-bound hydrogens on the glucose residue between 3 and 4 ppm (Fig. 11). For example, in the spectrum of isopropyl dGSL (**d56**) (Fig. 11A), the anomeric doublet is at 5 ppm, separate signals of the same intensity (1H each) are at 3.7 and 3.9 ppm (from the 2 H at carbon 6), while the remaining four glucose residue hydrogens form a complex multiplet around 3.4–3.6 ppm. Confirming that a GSL or dGSL is at hand depends on further analysis of coupling constants ("signal-splitting") of the glucose residue and testing

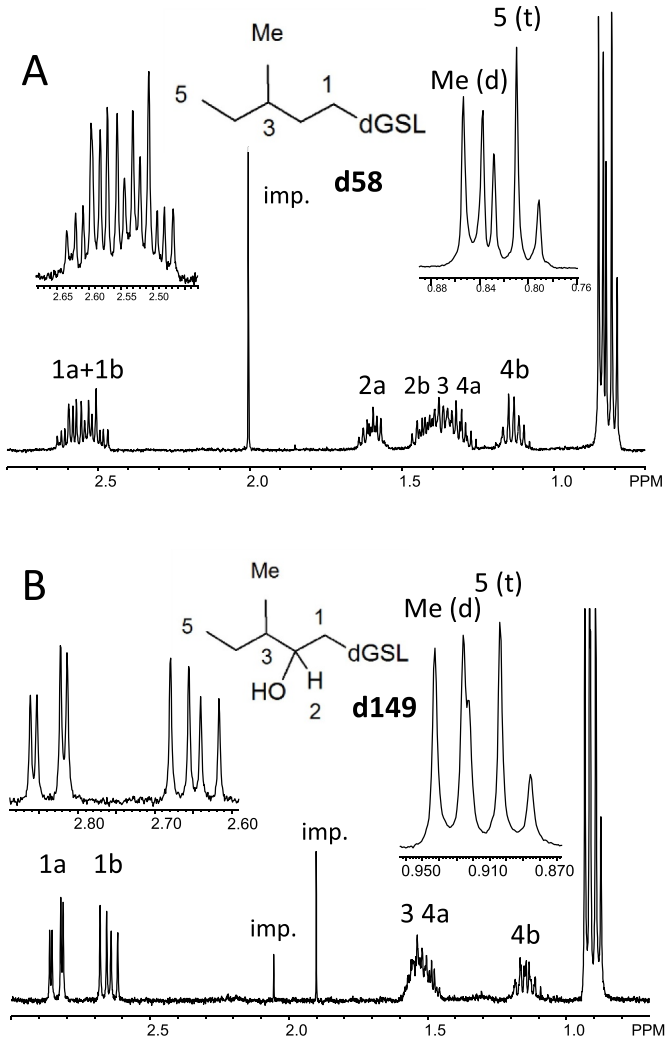


Fig. 12. Complex coupling within branched aliphatic side chains in desulfoglucosinolates (dGSLs), and simplifying effect of substitution. (A) Side chain region of the ^{1}H NMR spectrum of 3-methylpentyl dGSL (**d58**) in D_2O (Agerbirk et al., 2008, 2010a; Olsen et al., 2016). (B) Corresponding region of the spectrum of a hydroxyl derivative, 2-hydroxy-3-methylpentyl dGSL (**d149**) in D_2O (Olsen et al., 2016). It is evident that the coupling of the 1a and 1b signals in B is now first order, and that the signals of the position 2 protons have disappeared from the illustrated range of chemical shifts (to be found at 3.95 ppm). Occasional minor impurity peaks (imp) are generally recognizable by having peak areas less than unity.

the connectivity of the anomeric H to the thiohydroximate core. We return to this topic at the end of this section.

After having briefly recognized the glucose residue signals, attention is paid to side chain signals. In the ^{1}H NMR spectrum of isopropyl dGSL (**d56**) (Fig. 11A), anyone with a basic knowledge of NMR will recognize the side chain structure, based on the signal of the central H, split into 7 peaks due to the neighborhood of 6 protons on adjacent methyl groups ("$N+1$-rule"). Signals of the methyl groups themselves are further upfield. In agreement with the same rule, the signal of each methyl group is a doublet, reflecting that the neighboring C bears a single H. The two methyl groups do not have the same chemical shift since they are diastereotopic and have different chemical environment due to the influence of the nearby chiral glucose residue. This example illustrates the definite and predictable nature of NMR interpretation; the spectrum of hypothetic *n*-propyl GSL can be predicted with certainty to be completely different (from e.g. the $N+1$-rule). In contrast, the two possible isomers could not be distinguished by HPLC-MS without authentic standards of each.

In the spectrum of *N*-methoxyindol-3-ylmethyl dGSL (**d47**) (Fig. 11B), the glucose residue signals are somewhat different from those of **d56**, reflecting the magnetic influence from the aromatic ring. The anomeric proton is hidden under the solvent signal at 4.8 ppm (but can be resolved by 2D NMR), while other glucose residue signals are better separated. The side chain contains four independent spin systems. Four aromatic hydrogens between 7 and 8 ppm constitute one system forming two doublets and two triplets, and an isolated H at position 2″ forms a singlet in the same chemical shift range. The spin system consisting of the two geminal and diastereotopic CH_2 protons is responsible for the two doublets centered at 4.1 ppm, accidentally overlapping with the signal from the final spin system, the OCH_3 group. The intensity of all H-signals is proportional to the number of protons causing the respective signals. Although several hypothetic structures would be in agreement with this spectrum, only one known GSL (**47**) would fit an aromatic moiety with four neighboring and one isolated H, an isolated methylene group and an isolated methyl group. A combination of other results has confirmed the structure of **47** (Table 1).

The more complex structure in Fig. 11C could not at all be elucidated from the ^{1}H NMR spectrum alone, even though the spectrum of the 6′-isoferuloyl derivative **d132*R*** shows well separated signals. The glucose residue signals are well separated, suggesting an electron-withdrawing group in the 6′ position of that residue. A typical "ABX" spin system from –CH_2-CHOH– is well-separated (1a, 1b, 2). Two doublets with 1H intensity each and large coupling constant (much "splitting") at 6.1 and 7.5 ppm are evidence of the *trans* double bond in the isoferuloyl moiety, while two doublets with 2H intensity and smaller coupling constant (less splitting) at 6.75 and 7.25 ppm are evidence of the four *o*- and *m*-protons in the *p*-hydroxyphenyl group. 2D NMR, MS and chemical evidence (esterase-catalyzed hydrolysis to **d139*R*** and *trans*-isoferulic acid confirmed by HPLC) established the structure and 6′-connectivity of this GSL (Agerbirk and Olsen, 2011). The spectrum was generated from a rather low amount of analyte obtained by pooling from 40 HPLC collections (Agerbirk and Olsen, 2011). The neatness of the spectrum evidenced the remarkable achievable purity of dGSL preparations even when handling trace peaks (0.3 μmol/g dry wt. in case of **132*R***).

An additional type of routine spectrum, ^{13}C NMR, detects the carbon atoms in the GSL or dGSL. With a special version, known as DEPT-135, one can distinguish between primary, secondary and tertiary carbon atoms. In addition, 2D techniques are very powerful for establishing connectivity of atoms in the analyte. The 2D techniques are HSQC, identifying connections between adjacent H and C, HMBC, identifying connectivity between C and H through 2–3 bonds, NOESY, identifying proton proximity through space (e.g. the proximity of methyl group and indole H2 in **d138** (Agerbirk et al., 2001a), and COSY, introduced below. Further introduction to these NMR techniques is available in general organic chemistry textbooks, for two worked examples, see Supplementary Figs. S1 and S2.

Except for limited peak overlaps, the preceding structures could almost be interpreted by first order methods. However, peak overlap and complex coupling are the rule rather than the exception in the NMR of GSLs. This is well exemplified with the dihomo-Ile derived GSLs, a rather new structural group with three members (**58**, **141**, **149**) that have been discovered one by one in the past decade (Table 1). The first example is the spectrum of 3-methylpentyl dGSL (**d58**) (Fig. 12A). From MS, the side chain was known to be hexyl or a branched isomer, with multiple possibilities. Two methyl signals indicated one branch in the side chain. One methyl triplet revealed a terminal –CH_2-CH_3 (in contrast to –$CH(CH_3)_2$), and 2H intensity of the complex signal for H1a + H1b proved that the branching methyl group was not in that position. Hence, two side chains were possible, 2-methylpentyl and 3-methylpentyl. Detailed 2D NMR confirmed the latter (Agerbirk et al., 2008).

The specific argument for 3-substitution was not reported because **58** was wrongly considered known, in contrast to our present conclusion in Table 1. The specific argument was as follows: the position 1

CH_2 group was seen in COSY to couple with a second CH_2 group (in position 2), hence 2-substitution could be ruled out. Indeed, coupling from the branching methyl group was seen in COSY to a different proton (at position 3). A methylene group at position 2 and a methine group at position 3 was confirmed by DEPT-135. The complete set of spectra is reported in Supplementary Fig. S1.

In the 2-hydroxy derivative (**d149**), the upfield signals (Fig. 12B) were similar to those of **d58**, while the signals of position 1 were drastically simplified, showing a methylene group at *ca.* 2.6–2.9 ppm, with strong geminal coupling between H1a and H1b at the same C, and weaker vicinal couplings to H2 (compare to 1a and 1b in Fig. 11C). All the narrow doublets are seen to be "slanted" downfield (i.e. downfield peaks are slightly higher than the upfield peaks) indicating that the H2 signal is to be found in the downfield direction, i.e. to the left. Indeed, 2D NMR showed the H2 signal to be among the glucose residue signals (at 3.95 ppm). Hence, the 2-position of the hydroxyl group was ascertained. This position was confirmed by an aldol-cleavage-like fragmentation between C1 and C2 in MS/MS, characteristic for *β*-hydroxy substituted dGSLs (Olsen et al., 2016).

Another isomer was found in the same plant (Agerbirk et al., 2010a), and NMR-spectral analysis showed that the position of the hydroxyl group was different from that in **149**. The original elucidation used the dGSL (**d141**), but the NMR of the intact GSL (**141**), shown here for variation (Fig. 13A), is nearly superimposable. In this case, only one methyl group (5) is seen. It is a triplet, hence part of a CH_3–CH_2- moiety. A terminal position of the hydroxyl group was indicated by a –CH_2OH signal in ^{13}C NMR (DEPT-135 spectrum). Is the remaining side chain a pentyl, and if so, is the hydroxymethyl in position 1, 2 or 3? The 2D technique COSY (COrrelated SpectroscopY) (Fig. 13B) allows us to trace the connectivity of five pentyl carbons via the protons. They are progressively at higher chemical shift, counted from the terminal methyl triplet signal. Notably, the third of them has an extra cross-peak, showing direct neighborhood to the hydroxymethyl group at 3.55 ppm. Hence, the –CH_2OH branch is at the 3-position of a pentyl backbone. A complete set of NMR spectra of **141** is provided as Supplementary Fig. S2. We notice that three GSLs from the same plant were independently shown to exhibit a 3-methylpentyl skeleton, indicating probable biosynthesis from Ile, and with two different positions of the OH. Such conclusive structure elucidation is a hallmark of NMR.

We finally turn to confirmation of the structure of the carbohydrate moiety. We use **d40*R*** as an example (Fig. 14A); the interpretation of the side chain signals (a 5H multiplet in the aromatic region (not shown), a triplet (1H) at *ca.* 5.1 ppm and two double doublets (2H) at *ca.* 3.1 ppm) is trivial and left to the reader. Comparison of the diastereomers **40*R*** and **40*S*** (Sørensen, 1990) and the corresponding dGSLs (Agerbirk et al., 2001a) revealed an interaction between the glucose residue and the side chain, resulting in clearly different chemical shifts and coupling constants allowing distinction of (2*R*) and (2*S*) configuration by NMR. For example, the anomeric signal is at *ca.* 5.0 ppm (typically downfield of the solvent (HDO) signal) in **40*R*** and **d40*R***, but at ca. 4.8 ppm for the epimers **40*S*** and **d40*S*** (typically hidden in the solvent signal but revealed by COSY) (Agerbirk et al., 2001a, 2001b) (Fig. 14A). This NMR distinction is only possible because the epimeric pairs are diastereomers; enantiomers (like the OAT products) would have identical NMR spectra. This phenomenon allowed deduction of absolute configuration of derivatives like **50*R***, **131*R***, **131*S***, **132*S***, **139*R***, **139*S*** and **142*R*** (Agerbirk et al., 2001a, 2010b; Agerbirk and Olsen, 2011, 2012).

However, our aim here is to test critically whether the crowded multiplet at 3.4–3.9 ppm is due to a glucopyranose residue, as opposed to some other sugar that could hypothetically (Section 5.1.7.) be present in place of glucopyranose. The evidence missing is the coupling constants (*J*) of the glucose residue protons (the "splitting" of the individual signals, in the unit Hz). In the glucopyrano chair conformation (a unique case among all hexoses), hydrogens 1–5 are axial and close to antiparallel. As coupling depends on the "dihedral angle", this results in three coupling constants close to 7–9 Hz. For diastereomeric sugars, at least one coupling constant would be lower due to at least one equatorial H. What we need is the resolution of the overlapping triplets, to reveal the coupling constants. The so-called *J*-resolved (*J*RES) spectrum allows this, confirming the sugar moiety to be glucopyranose (Fig. 14B). We still have not distinguished the usual D-glucose from the alternative extremely rare L-glucose. The myrosinase enzyme from *S. alba* can probably do this for us, since this chiral reagent binds efficiently to the entire *β*-D-glucopyrano moiety (Burmeister et al., 1997). (However, critical testing of the chiral specificity of this reagent should ideally be tested and published using synthetic analogs). Indeed, incubation of isolated **40*R*** with myrosinase results in rapid hydrolysis (Agerbirk and Olsen, 2012). In conclusion, the constant part of **40*R*** contains a *β*-D-glucopyranosyl unit. Equivalent results have been found for all tested GSLs. Final confirmation of the constant GSL or dGSL structure is done

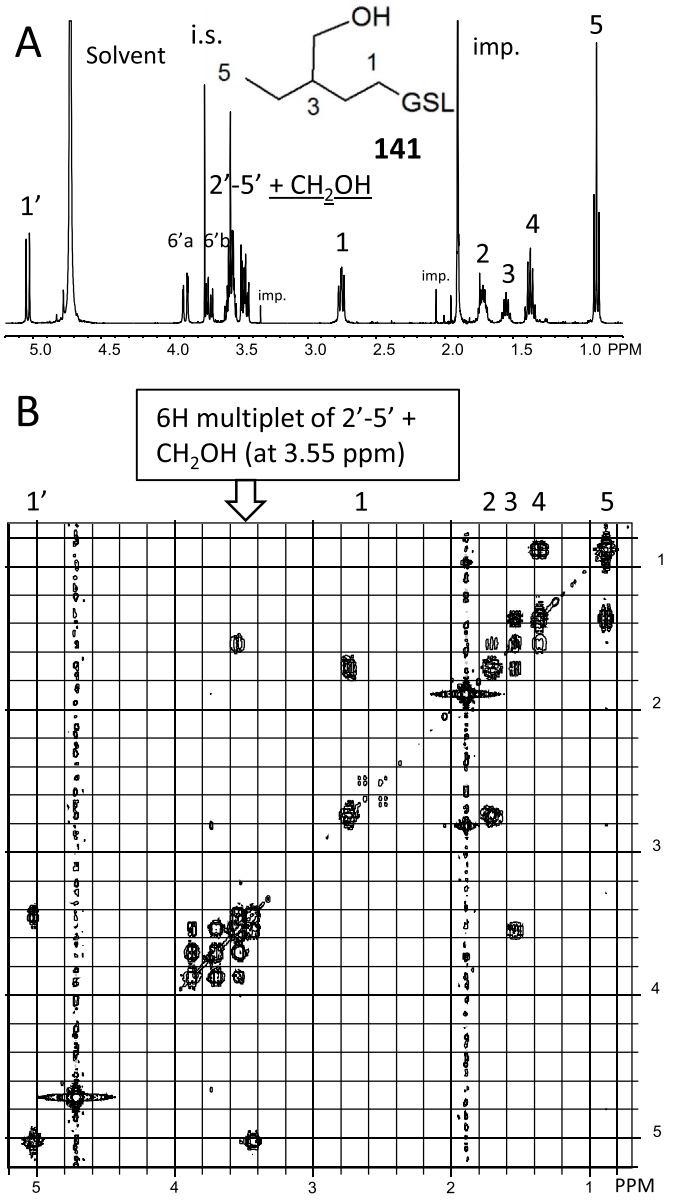


Fig. 13. The usefulness of the COSY spectrum for establishing connectivity, and an example of NMR of an intact glucosinolate (GSL). (A) The ^{1}H NMR spectrum of 3-(hydroxymethyl)methylpentyl GSL (**141**) in D_2O. A major impurity of acetate from the ion exchange isolation is seen. (B) The corresponding COSY spectrum. Signals along the diagonal correspond to the 1D spectrum. Whenever protons are found at the same or neighboring carbons, a "cross peak" can be seen with the x-coordinate of the one signal and the y-coordinate of the other. Original data by the authors (CEO and NA, Sections 6.1 and 6.2).

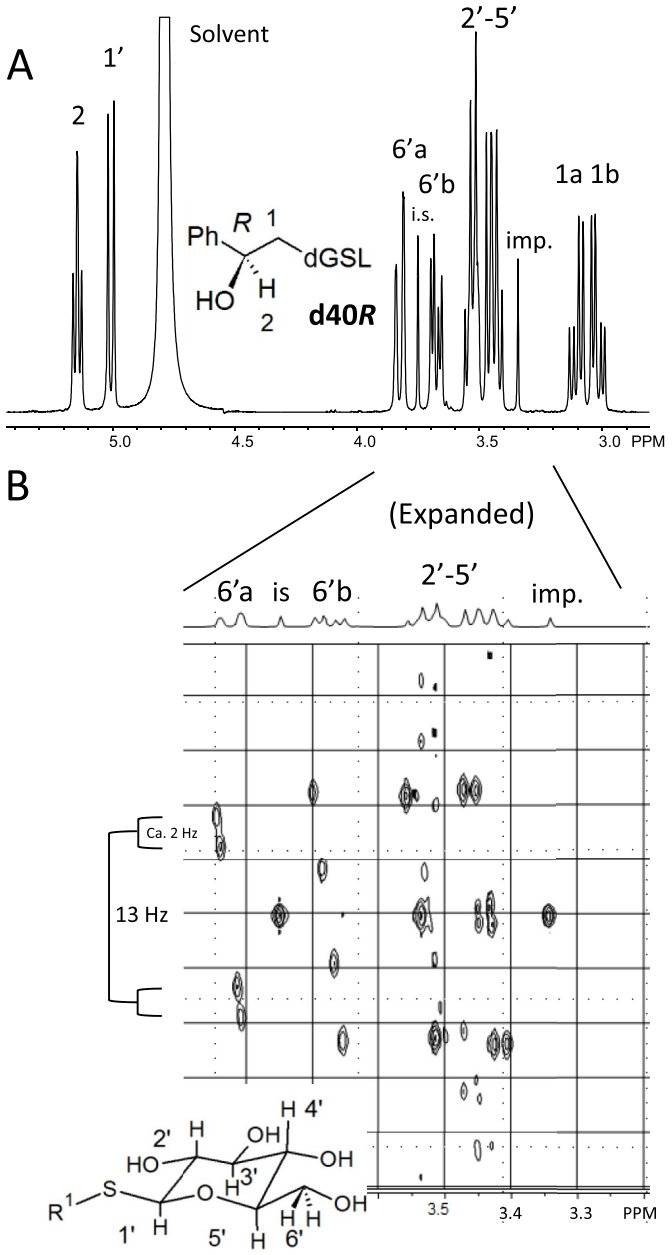


Fig. 14. The usefulness of the *J*-resolved (*J*RES) spectrum for resolving overlapping signals. (A) The upfield part of the ^{1}H NMR spectrum of **d40*R*** in D_2O and dioxane as internal standard (i.s.). A minor impurity (imp.) of MeOH from the HPLC isolation is seen. (B) In the *J*RES ^{1}H NMR spectrum, the splitting of signals is plotted (almost) perpendicular to the chemical shift x-axis, allowing resolution of signals at nearly identical chemical shift. The known coupling constants of the 6′a double doublet signal can be used for estimating coupling constants in the newly revealed signals. Three obvious triplets with coupling constant (*J*) of *ca.* 8 Hz can be seen. In addition, the complex multiplet of the 5′ proton is visible as a line of low intensity signals. Singlets from the i. s. and imp. do not show coupling, and all signals are seen at the same chemical shift coordinate as in the 1D spectrum. Unpublished results from identification of **40*R*** (Agerbirk et al., 2001a).

efficiently by testing for long-range coupling (in an "HMBC-spectrum", Supplementary Figs. S1 and S2) between the anomeric H and the thiohydroximate C (found at *ca.* 161 ppm for GSLs and 152 ppm for dGSLs). This connectivity for the anomeric H is characteristic of a GSL or dGSL. The invariant chemical shift of C0 in intact GSLs was an early argument for all GSLs having (*Z*)-configuration (Olsen and Sørensen, 1981) as demonstrated by X-ray crystallography only for **73** and **107** (Table 1, Fig. 1).

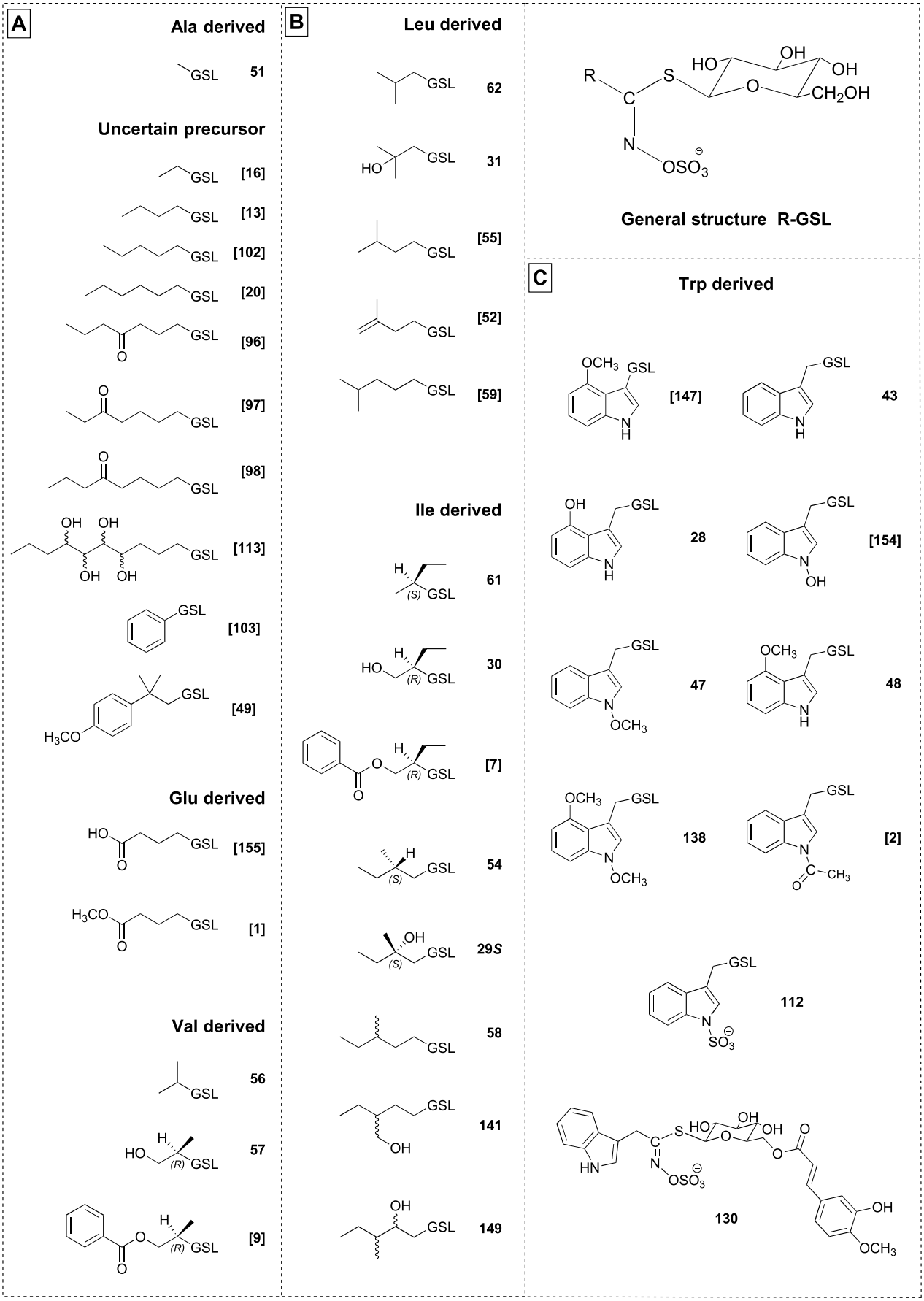
Several authors have prepared useful tables of NMR spectral data for a range of GSLs and dGSLs: for **11**, **12**, **22, 23, 24**, **40**, **43**, **51**, **73**, **105**, **107**, **109** (Olsen and Sørensen, 1981); for **11**, **12**, **23**, **24*S***, **d24*S***, **43**, **73**, **82**, **84** and **107** (Cox et al., 1984), for synthetic **d12**, **[d13]**, **[d16]**, **d51**, **d56**, **d62**, **d101**, *n*-propylGSL and plant-isolated **d11**, **d23**, **d24**, **d28**, **d40*R***, **d43**, **d47**, **d48**, **d64**, **d73**, **d84**, **d105** (Kiddle et al., 2001); for **d5**, **d117**, **d125**, **d126**, **d127**, **d137** (Reichelt et al., 2002); for **40*R***, **40*S***, **d40*R***, **d40*S***, **d139*R***, **d139*S*** (Agerbirk et al., 2001a); for **d28**, **d43**, **d48**, **d138** (Agerbirk et al., 2001b); for **d11**, **d12**, **d23**, **d24*R***, **d46**, **d56**, **d58**, **d61**, **d84**, **d105**, **d140**, **d143** (Agerbirk et al., 2008); for **d30**, **d57**, **d58**, **d141** (Agerbirk et al., 2010a); for **50*R***, **140**, **143** (Agerbirk et al., 2010b); for **131*S***, **131*R***, **d43**, **d105**, **d129**, **d130**, **d131*R***, **d131*S***, **d132*R*** (Agerbirk and Olsen, 2011); for **d12**, **d54**, **d62**, **d64**, **d66**, **d69**, **d92**, **d101**, **d107**, **d149** (Olsen et al., 2016); for **d15**, **d22**, **d114**, **d151**, **d152** (Pagnotta et al., 2017; for **63**, **d29*S***, **d57** and both intact and desulfated **11**, **12**, **23**, **24*R***, **24*S***, **40*S***, **45**, **64**, **73**, **83**, **84**, **105**, **107**, **110** (Ibrahim et al., 2018). References for individual compounds are also provided in Table 1. A recent report discussed modern interpretation of coupling using the PERCH software and demonstrated several cases of long range coupling (Ibrahim et al., 2018). In particular, the anomeric H in benzylic GSLs and dGSLs can exhibit a complex multiplicity, which was shown by COSY to be due to additional coupling to H3′ (Müller et al., 2015) and was in accordance with PERCH calculations (Ibrahim et al., 2018).

Journals nowadays allow publication of actual NMR spectra as figures or supporting information; we strongly recommend this possibility as it allows critical evaluation and re-interpretation if relevant. Examples of such figures are: for **24*R***, **24*S***, **40*R***, **40*S***, Sørensen (1990); for **61**, Dauvergne et al. (2006); for **d129**, **d130**, **d131*R***, **d132*R***, Agerbirk and Olsen (2011); for **40*R***, **66**, **105**, Agerbirk et al. (2014); for **d139*S***, **d140**, **d148**, Agerbirk et al. (2015a); for **110**, Müller et al. (2015); for **d23** and **d110** (2D spectra), de Graaf et al. (2015); for **d12**, **d43**, **d47**, **d54**, **d56**, **d58**, **d61**, **d62**, **d66**, **d69**, **d101**, **d107**, **d138**, **d141**, **d149**, Olsen et al. (2016); for **62**, Sirinut et al. (2017); for **45** (worked example incl. 2D spectra), **105**, **d105**, Ibrahim et al. (2018).

2.3. Natural glucosinolates characterized since 2011

Since the last critical review of GSL structures, ending in 2011, additional structures have been published. In accordance with our inclusion of some partially characterized GSLs among numbers up to 120, we also include some partially characterized GSLs from among those recently published. However, the required level of characterization is generally higher for structures reported after 2000. Publication standards were set too low for numbers up to 120, and the increasing number of claims from superficially interpreted HPLC-MS analyses would make it unrealistic and of no scientific value to keep track of them all. Both classical and novel structures are illustrated together in Fig. 15, so the classification based on amino acid precursor is introduced here.

Since much of current biology is based on molecular genetics and expression analysis of biosynthetic enzymes, we have arranged the GSLs according to apparent biosynthetic precursor amino acid (Fig. 15), judged by side chain structure and biosynthetic evidence from *A. thaliana*. Historical experience (Section 1.1.2.) shows that apparent biosynthetic precursors as judged from molecular skeletons are usually confirmed when tested, making this classification reasonable. Hence, most structures are grouped according to side chain structure as probably derived from (Section 1.1.2.): Ala, Glu (via homoGlu), Val, Leu (=homoVal) and homologs, Ile and homologs, Trp, Phe/Tyr and homologs, Met and homologs, and finally selenoMet and homologs. In the case of **[147]** (Fig. 15C), biosynthesis seems to be indirectly from Trp via 4-methoxyindolylGly, so also this suggested GSL can be viewed as Trp-derived (Section 4.4.). Since the biosynthesis of GSLs with terminal hydroxyl groups is well known to start with Met in *A. thaliana*



(caption on next page)

Fig. 15. Structures of all documented glucosinolates (GSLs) by mid-2018, including some structures [in brackets] that are only partially characterized but with the available evidence pointing at one single structure. The GSLs are grouped by apparent amino acid precursor according to criteria presented in Section 2.3. The distribution of GSLs among the nine panels, A-I, is indexed in Table 2.

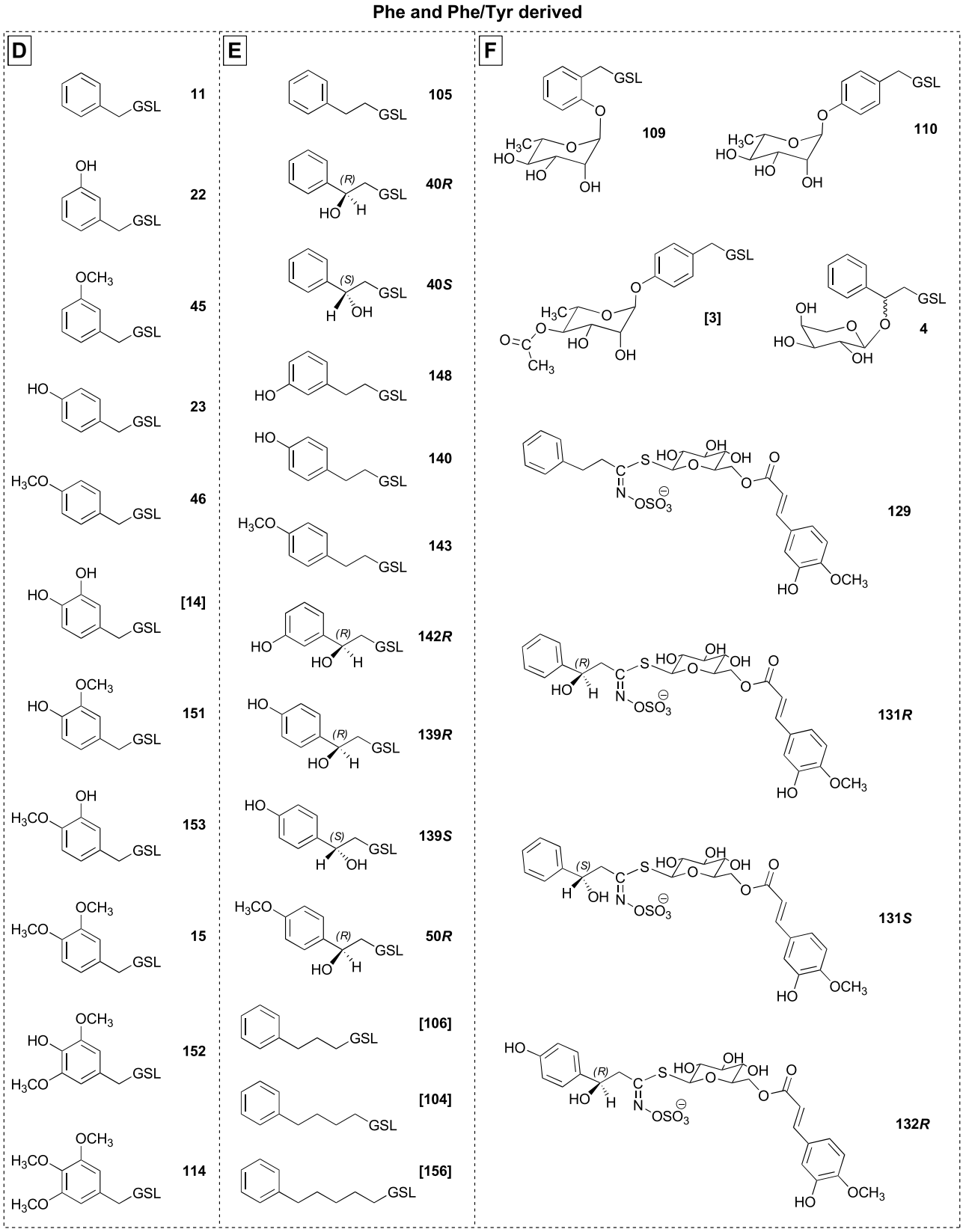


Fig. 15. (*continued*)

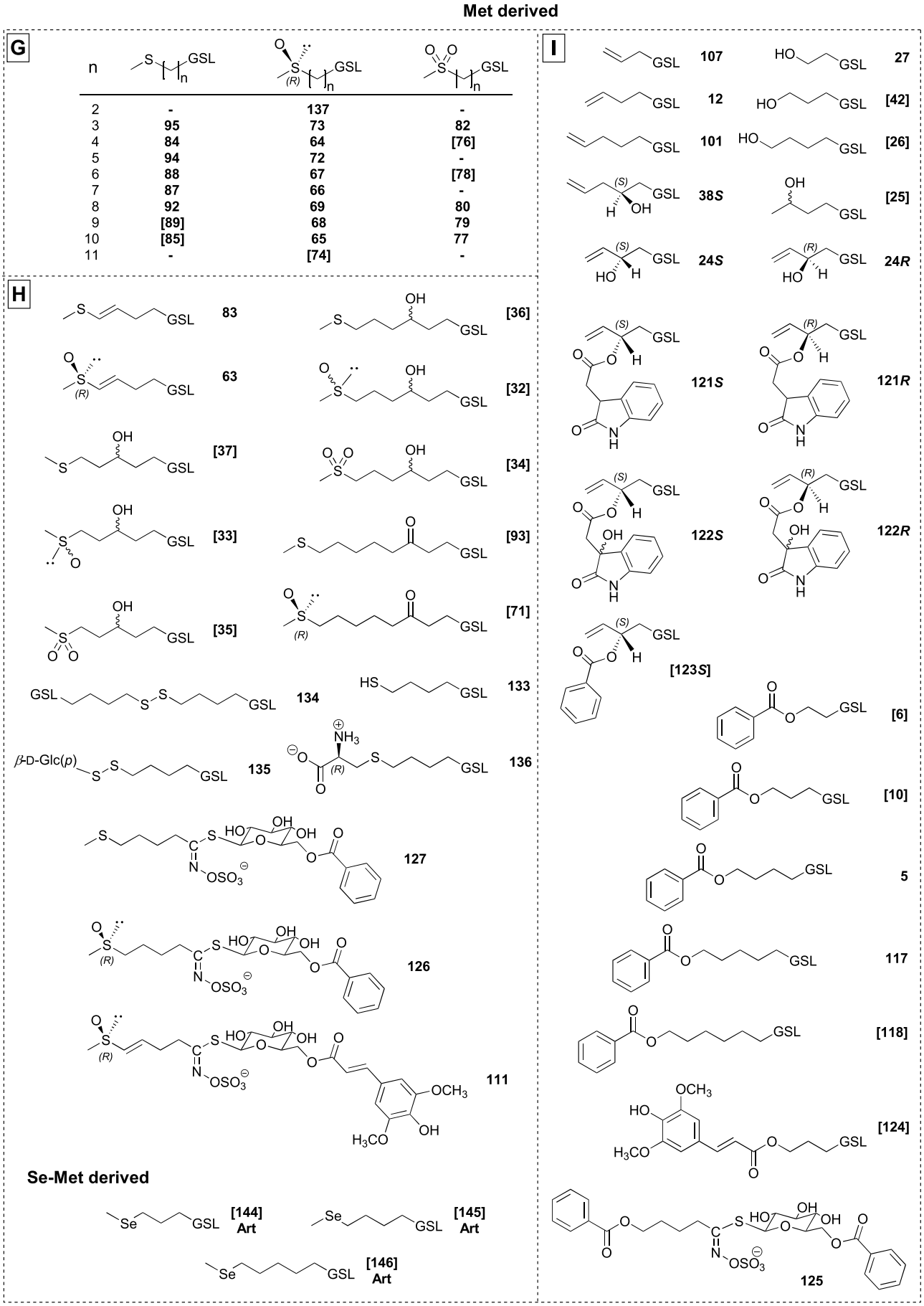


Fig. 15. (*continued*)

(Fig. 6) (Kliebenstein et al., 2001a; Sønderby et al., 2010a, 2010b), those are attributed to Met rather than to Ser. As a similar mechanism would allow insertion of hydroxyl groups at the ω-1 position, one such case, **[25]** (Fig. 15I), was also classified as Met-derived for simplicity, but this classification is tentative. Since GSLs with a terminal mercapto group and similar structures (**133–136**, Fig. 15H) co-occur with GSLs derived from Met (Cataldi et al., 2007), the former are likewise listed as Met-derived rather than as Cys-derived for simplicity; also these classifications are tentative. Finally, structures that cannot in a simple way be explained from usual amino acids with chain elongation, or which could equally well be derived from a number of amino acids (e.g. Ala or Met), are grouped under "uncertain precursor".

Since the last critical review of GSL structures (Agerbirk and Olsen, 2012), and until mid-2018, the following new GSLs have to our knowledge been reported with reasonable evidence for one specific structure (treated in quasi-chronological order, keeping the numbers used by Olsen et al., 2016).

A new but expected structure, 4-methoxyphenethyl GSL (**143**, Fig. 15E), was isolated and used for studying GSL detoxification in caterpillars of the small white cabbage butterfly, *Pieris rapae* L. In addition to the previously known nitrile formation from GSLs in general, caused by the nitrile specifier protein in the caterpillars (Wittstock et al., 2004), the frass collected after ingestion of isolated **143** revealed several additional detoxification steps (hydroxylation plus demethylation followed by sulfation) (Agerbirk et al., 2010b).

Based on identification of apparent GSL hydrolysis products, three Se-Met derived GSLs of the general type ω-(methylseleno)alkyl GSL were suggested: **[144]**, **[145]** and **[146]** (Fig. 15I) (Matich et al., 2012). Because of current agronomic attempts at providing vegetables enriched in bioavailable selenium, Se-containing GSLs can be expected to receive much attention in the future (Wiesner-Reinhold et al., 2017), but see Tian et al. (2018). The enzymology, analytical chemistry and physiology of such GSLs will also be exciting. The structures are further discussed in Section 2.6.

The remarkable 4-methoxyindol-3-yl GSL (**[147]**, Fig. 15C) (a genuine indolyl GSL as opposed to an indolylmethyl GSLs) is yet only tentatively demonstrated, while the corresponding ITC and a 5-hydroxy derivative are well-characterized phytoalexins (Pedras et al., 2007). After extensive and careful experiments, biosynthesis of the ITC through the hypothetical unstable GSL **[147]** was deduced (Pedras and Yaya, 2013; Pedras et al., 2016). This research work directly connects deduced GSLs and phytoalexins, and suggests the new indolyl ITCs to be ultimately biosynthetically derived from the parent indole GSL **43** (Pedras and Yaya, 2013). It is difficult to evaluate this research work because of the atypical methods used due to the apparent instability of the suggested GSL. If this well-argued suggestion can be confirmed by biochemical evidence, it is close to a revolution in our understanding of the biochemistry of Trp-derived GSLs in plants (reviewed by Agerbirk et al., 2009). The 5-hydroxy derivative, 5-hydroxy-4-methoxyindol-3-yl ITC, is also the first confirmed case in GSL biochemistry of a 5-substituted indole derivative, but the discoverer has so far not suggested the existence of the corresponding GSL, hence no number corresponding to this documented ITC is proposed here.

The structural diversity of GSLs in the "eco-model" crucifer *Barbarea vulgaris* R. Br. (wintercress) was further illustrated by identification of the *m*-hydroxyphenethyl GSL (**148**, Fig. 15E), which was isolated from a natural variant with low degree of 2-hydroxylation (Agerbirk et al., 2015a). The structure confirmed that phenolic GSLs in *Barbarea* can be derived from Phe (several *para*-hydroxy derivatives could also be derived from Tyr). The structure was also interpreted to suggest that GSL secondary modifications in *B. vulgaris* are due to a combinatorial system of enzymes with less than absolute substrate specificity, as the same *meta*-substitution had previously been found in **142*R*** (Fig. 15E) in plants with high degree of 2-hydroxylation.

A new β-hydroxylated Ile-derived GSL, 2-hydroxy-3-methylpentyl GSL (**149**), was identified in a crucifer species already known to form the isomer **141** and the expected common precursor **58** (Fig. 15B) (Olsen et al., 2016). An intriguing feature of the new isomer was its presence in roots but absence in shoots, which were dominated by **141**. Future investigations could focus on the expected contrasting hydrolysis products of a β- vs. a δ-hydroxylated GSL, on the biosynthesis (catalyzed by GS-OH enzymes or another family?) and the biological effects. During the writing of this review, we became aware that the entire group of dihomo-Ile derived GSLs has only recently been discovered. Although the two hydroxy derivatives **141** and **149** have so far only been found in one species (*C. pratensis*), the parent structure **58** has been conclusively detected also in two further distantly related genera (Agerbirk et al., 2008), and may have been overlooked elsewhere such as suggested "*n*-hexyl GSL" in several reports (Bennett et al., 2004; Mithen et al., 2010).

The impressive number of documented derivatives of benzyl GSL was increased by two with the identification of 4-hydroxy-3-methoxybenzyl GSL (**151**) and 4-hydroxy-3,5-dimethoxybenzyl GSL (**152**) (Fig. 15D) (Pagnotta et al., 2017). The structures were used to draft a hypothetical biosynthetic route to 3,4,5-trimethoxybenzyl GSL (**114**) that occurs in the same plant (the invasive *Lepidium densiflorum* Schrad., common pepperweed). Detection of benzyl GSL suggested Phe to be the precursor. The new **152** was the dominating GSL. As expected for a *p*-hydroxybenzyl GSL, no ITC was detected. In contrast to the expected benzylic alcohol product, a substituted benzaldehyde (syringaldehyde) appeared to be the major product in crushed tissue (Section 4.4). The required oxidation from alcohol to aldehyde was not further investigated. Previous carefull investigations found different GSLs (Montaut et al., 2017), suggesting a GSL polymorphism in the species.

As part of a long tradition of investigating poorly known, potentially useful species, 3-hydroxy-4-methoxybenzyl GSL (**153**, Fig. 15D) was identified from a Chinese tree of the family Akaniaceae (Montaut et al., 2015). Interestingly, an isomer (**151**) was discovered a few years later in a distant species, see above. The quite high frequency of discovery of novel GSLs when rarely investigated material is studied, underlines how limited our knowledge of GSL biodiversity still is. A pair of isomers such as **151** and **153** suggests the existence of biosynthetic enzymes that could be of interest for genetic design of new GSL profiles in crops. For instance, **151** would form vanillin in case of aldehyde formation as seen for **152**.

The previously anticipated (Pfalz et al., 2011) *N*-hydroxy intermediate in indole GSL biosynthesis, **[154]** (Fig. 5A) was confirmed by MS of the intact GSL and MS2 of the dGSL, exhibiting a critical loss of 17 Da (OH radical) (Pfalz et al., 2016). Genetic evidence supported the identification (Fig. 5). However, the authors were not able to isolate neither the GSL nor the dGSL, which were apparently unstable, so the structure is regarded as tentative until NMR evidence is available. The previous evidence was reviewed by Agerbirk and Olsen (2012), and the evidence published since then (Pfalz et al., 2016) resolved the specific uncertainty pointed out in 2012.

The existence of an apparently Glu-derived GSL (3-carboxypropyl GSL **[155]**, Fig. 15A), was suggested from a classical type of partial evidence: GC-MS identification of an ITC, including direct comparison with an authentic reference (Radulović et al., 2011). The existence of a Glu-derived GSL in this genus (*Erysimum*) was not unexpected, as Glu-derived **[1]** was known, suggesting that the genus warrants further study. This deduced GSL was concluded to be tentative in a previous review (Agerbirk and Olsen, 2012) because evidence for the GSL itself was lacking. With the present inclusion in the list of GSLs deduced from ITCs, it has been inserted in the numbering system albeit in brackets. **[155]** is included here because modern GC-MS comparison with an authentic reference is considered a strong tool for the identification of the ITC (Section 5.2.).

From similar tentative evidence, the existence of the Phe-derived 5-phenylpentyl GSL (**[156]**, Fig. 15E) was suggested based on GC-MS identification of 5-phenylpentyl ITC in a horseradish autolysate (Dekić et al., 2017). Again, the existence of this GSL needs confirmation.

Similar tentative evidense for the two lower homologs **[106]** and **[104]** (Fig. 15E) was also supplied (Dekić et al., 2017). The literature of horseradish GSLs and ITCs is highly fragmented and scattered (Grob and Matile, 1980; Agneta et al., 2012, 2014; De Maria et al., 2016; Ciska et al., 2017; Dekić et al., 2017). An overview of suggested GSL diversity in horseradish was given by Dekić et al. (2017). Future papers should likewise take into account the previous literature, aiming at a critical evaluation.

2.4. Glucosinolates characterized satisfactorily

Under this heading, only GSLs listed without brackets in Table 1 and Fig. 15 are covered, i.e. GSLs characterized by NMR and MS of the glucosidic form (as GSL or dGSL derivative). With this condition, the entire GSL structural biodiversity is fully compatible with biosynthesis of aglucone carbon skeletons from Ala, Val, Leu, Ile, Trp, Phe and Met, as well as chain-elongated Ile, Phe and Met. Tyr is not mentioned in this listing, and neither in the following, as it can be regarded as *p*-hydroxylated Phe. From those carbon skeletons, further diversity is known in the form of six main variations:

1) Oxidation of sulfur in Met-derived side chains to sulfoxides ("sulfinyls") and sulfones ("sulfonyls");
2) Terminal unsaturation after loss of methylated sulfur (via a methylsulfinyl intermediate, Fig. 6);
3) Hydroxylation of aliphatic and aromatic carbons;
4) Further methylation of side chain oxygens;
5) Glycosidation of side chain oxygens to form a rhamnoside or arabinoside;
6) Esterification of side chain and/or glucose residue (6′ position only) by carboxylic acids (benzoic acid, sinapic acid, isoferulic acid and oxindol-3-ylacetic acid derivatives).

Further atypical diversity relies on substitution of indole GSLs and 4-mercaptobutyl GSL (**133**, Fig. 15H). The indole GSLs are substituted at the indole *N* by sulfonation (**112**) or methoxylation (**47**) (Figs. 5A and 15C). If the mercaptoalkyl GSL **133** is indeed derived from Met, demethylation may be involved in the biosynthesis, and further unusual steps could occur in the formation of a small group of derivatives of **133**: a symmetrical disulfide (**134**), a non-symmetrical disulfide with 1-thioglucose (**135**), and a non-symmetrical sulfide connected to a group suggested to be derived from Ser (**136**) (Fig. 15H).

Absolute configurations of stereogenic centers in the carbon skeleton are, as far as we know, identical to those in the deduced amino acid precursors, and natural (biosynthesized) methylsulfinylalkyl GSLs are, as far as we know, always *R*-configured (Table 1, see **64**). Hence, there is no need for indicating these configurations in the numbers. In contrast, methylsulfinylalkyl GSLs produced by controlled-chemical oxidation of corresponding methylsulfanylalkyl GSLs, e.g. by H_2O_2 or spontaneous oxidation, are in the form of epimeric pairs with respect to the chirality of the sulfoxide (Iori et al., 1999). However, additional biological variation arises from biosynthetic hydroxylation of side chain carbons in either *R*- or *S*-configurations, resulting in six documented epimeric pairs (e.g. **24*R***, **24*S***) (Fig. 15E + F + I) and several cases where a missing epimer is to be expected but not yet discovered (e.g. **38*S***) (Fig. 15I). For this reason, our numbering system includes an added *S* or *R* for GSL stereoisomers caused by hydroxylation of a side chain carbon, but not for those chiral side chains that are a mere consequence of a chiral amino acid precursor not expected to vary naturally.

The combination of these kinds of structural variation produces 88 GSLs considered satisfactorily characterized by the criteria chosen for this review (Table 1, Section 2). That is, with modern spectroscopic data (NMR and MS) showing one specific skeletal structure, but not always the absolute configuration at chiral centers. This variation is far from being evenly distributed over biosynthetic groups. Indeed, 42 of them can be derived from Met and chain elongated homologs (but Met as precursor is speculative for **[25]** and **133–136**). In addition, 27 can be derived from Phe and chain-elongated homologs. Furthermore, 7 can be derived from Ile and chain-elongated homologs, while the remaining can be derived from standard amino acids without chain elongation: Trp (7), Leu (2), Val (2) and Ala (1) (Fig. 15).

2.5. Glucosinolates characterized partially

A total of 46 natural GSLs are listed as suggested with reasonable but highly variable evidence, yet not fully characterized. In continuation of the count in the previous section, the number of reasonably characterized natural GSLs seems to be in the range 88–134, and for narrowing the range complied here, there is a need for modern investigations. In addition, three selenium-containing GSLs, so far classified as artificial, are partially characterized.

The GSLs classified as not fully characterized in this review form a very heterogeneous group in terms of degree of characterization. The authors acknowledge that some of them could have been put in the group of fully characterized GSLs by using other, also scientifically based criteria. However, it is interesting to note that the less than fully characterized GSLs include characteristic structural groups. Hence, in order to better understand the entire structural variation of natural GSLs, those subgroups warrant future investigation. Six groups of insufficiently characterized GSLs seem particularly interesting:

(1) Both apparent Glu-derived GSLs **[1]** and **[155]** (Fig. 15A);
(2) All suggested GSLs apparently derived from chain-elongated Leu (Fig. 15B);
(3) The three homologs derived from Phe with more than one chain elongation (**[104]**, **[106]**, **[156]** (Fig. 15E);
(4) All suggested Met-derived GSLs with a hydroxyl or keto group in the 3-position (**[25]**, **[32]**-**[37]**, **[71]**, and **[93]**), a group that has received surprisingly little attention after the original reports (Fig. 15H);
(5) The well-characterized *ω*-methylsulfonylalkyl GSLs form two subgroups, a single short-chain GSL (**82**) and a group of three long-chain GSLs (**80**, **79** and **77**). Between these, some possible structures are missing, while others (**[76]** and **[78]**) are not fully characterized. This pattern could indicate a true gap between two biosynthetic groups (Fig. 15G);
(6) A series of GSLs with unbranched side chains, listed under "Uncertain precursor" (Fig. 15A). This group could also have been classified as potentially Ala derived (after chain elongation) or Met derived (after removal of the methylsulfanyl moiety).

We recommend future critical investigation into the existence of selected members of these groups, in order to improve our understanding of the natural structural variation of GSLs. The remaining insufficiently characterized GSLs are of scattered occurrence. Some of them seem to be an expectable consequence of very low levels typically found. This could be the case for the suggested sinapoate **[124]** (Fig. 15I) and acetate **[3]** (Fig. 15F) as well as for the longest chain members of the homologous series of *ω*-(methylsulfanyl)alkyl GSLs and *ω*-(methylsulfinyl)alkyl GSLs (Fig. 15G). Similarly, quite a few benzoyl derivatives of hydroxyalkyl GSLs are insufficiently characterized (**[6]**, **[7]**, **[9]**, **[10]**, **[118]**), while others are well characterized (**5**, **117**, **125–127**); so the evidence for benzoylated GSLs in general is convincing. Likewise, two *ω*-hydroxyalkyl GSLs (**[26]** and **[42]**, Fig. 15I) are insufficiently characterized, whereas the benzoate **5** (Fig. 15I) is fully characterized. As **5** is a derivative of **[26]**, the definite structure of **5** provides biochemical support for the proposed existence of **[26]** and *ω*-hydroxyalkyl GSLs in general (Fig. 6). In addition, the apparently rare suggested *N*-acetyl derivative **[2]** (Fig. 15C) of the parent indole GSL **43** needs further research. Although not fully characterized, some unstable Trp-derived GSLs (**[147]**, **[154]**) both appear as intermediates of increasingly well-characterized biosynthetic pathways (Fig. 5,

Section 4.4.).

Finally, a GSL with an unexpected carbon skeleton, **[49]** (Fig. 15A), lacks conclusive evidence. This structure has been reported only once (El-Migirab et al., 1977) (deduced from the ITC), and a careful modern re-investigation of the same plant species could not confirm its existence (De Nicola et al., 2012). Hence, although the original report contains reasonable characterization of the corresponding ITC, there is reason to regard this exceptional structure (with a carbon skeleton that cannot formally be derived from a standard amino acid) with skepticism. However, to biosynthesize this interesting suggested structure from dihomoPhe one would simply require a biochemical C-methylation, which is a conceivable biosynthetic step. The poorly evidenced phenyl GSL (**[103]**, Fig. 15A) (deduced from the ITC) would also require a new biosynthetic step. As synthetic **[103]** is available, the suggested natural occurrence of **[103]** should be tested before any further speculation.

2.6. Glucosinolates only detected in manipulated plants

Selenoglucosinolates were originally defined as compounds in which a Se atom replaces the S atom in the anomeric position in the GSL framework, convertible through enzymatic hydrolysis into isoselenocyanates (Bertelsen et al., 1988). Formation of isoselenothiocyanates is a significant deviation from properties of GSLs (Fig. 16). SelenoGSLs sensu Bertelsen et al. (1988) are by definition not GSLs (Section 1.1.). However, the presence of Se in side chains is fully compatible with the definition of GSLs, and has been suggested based on identification of apparent hydrolysis products from plants artificially fed with high levels of selenate (Matich et al., 2012). GSLs containing Se in the side chain (Fig. 15H) could be conveniently named selenoMet-derived GSLs to avoid confusion with selenoGSLs sensu Bertelsen et al. (1988). This would be appropriate because the former yield ITCs, not isoselenocyanates as hydrolysis products.

Since the plants studied by Matich et al. (2012) were usual *Brassica* crop varieties, the formation of GSLs with Se in the side chain can be viewed as a natural response to high selenate levels. Their detection in this pioneering work might imply that similar compounds could be formed at more usual selenate levels, but this possibility would be dependent on the precise properties of the enzymes involved. A further Se-analog of 4-(methylsulfinyl)butyl GSL (**64**, Fig. 15G) was more recently tentatively suggested from HPLC-MS/MS experiments (Matich et al., 2015; Wiesner-Reinhold et al., 2017), and the Se concluded to not be in the constant part of the envisioned GSL. In addition, a Se analog of phenethyl GSL (**105**, Fig. 15E) was tentatively suggested by HPLC-MS, but in this case, Se could not be envisioned to be in the side chain, so a selenoGSL sensu Bertelsen et al. (1988) could be at hand (Matich et al., 2015). The latter suggestions have not yet been given numbers in Table 1, as more solid evidence is needed.

When this review was initiated, the authors anticipated that novel

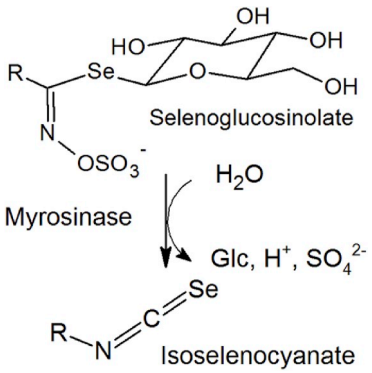


Fig. 16. Definition of the term selenoglucosinolate, precursor of an isoselenocyanate, according to the discoverers, Bertelsen et al. (1988).

"artificial" GSLs due to genetic engineering would be abundant in the literature, but only a few tentatively ascribed "artificial plant GSLs" emerged from the literature search. As 4-cysteinylbutyl GSL (**136**, Fig. 15H) has apparently only been detected under rather artificial conditions (Table 1), it could be classified as potentially "artificial" or perhaps generally overlooked for analytical reasons or restricted to very specific physiological conditions. However, this case does not involve genetic manipulation. From *A. thaliana* mutants deficient in the final methylation step in the biosynthesis of *N*-methoxyindol-3-ylmethyl GSL (**47**), some apparent detoxification products of *N*-hydroxyindol-3-ylmethyl GSL (**[154]**) were detected and partially characterized by HPLC-MS/MS and UV (Pfalz et al., 2016). One could hypothesize that rare, tentatively identified indole GSLs such as **[2]** and **[112]** (Fig. 15C) are consequences of similar mutations in the biosynthesis of **47**.

It is likely that more "artificial plant GSLs" will be encountered in the coming years. We will leave their classification to future reviewers. From one perspective, they are not part of the documented natural evolution of GSL diversity. From another perspective, some of them might illustrate likely metabolites that would be formed also naturally after specific mutations.

2.7. Examples of additional suggested glucosinolates

Examples of suggested GSLs with insufficient evidence up to 2011 were generally discussed in a previous review (Agerbirk and Olsen, 2012). Multiple GSLs have been suggested without detailed reasoning from MS/MS or other data since then, and not all can be discussed here. Selected examples are summarized here in quasi-chronological order, with the purpose to point out specifically the lacking pieces of evidence or even misinterpretation.

Two reports were accidentally not reviewed in the previous review, but are covered here. From *Hesperis matronalis* L. (dame's rocket), complex derivatives (containing an apioside residue) of 3,4-dihydroxybenzyl GSL (**[14]**) were claimed (Larsen et al., 1992) and reported to be specific feeding stimulants for a monophagous beetle. The claimed GSLs were listed as "119" and "120" by Fahey et al. (2001), who noted that no data supporting the claim were ever reported. Later, Bennett et al. (2004) reported HPLC-MS peaks with the expected *m/z* values and loss of sulfate. Although this observation is far from sufficient evidence for the claimed structures, re-investigation of this plant species is relevant.

A report by Sarg et al. (1995) was also missing in the previous review. From *Ochradenus baccatus* Delile (taily weed, Resedaceae), one known (**22**) and three possibly novel GSLs were tentatively reported from wet chemistry characterization of isolated GSLs and from MS, UV and IR of products from acid or enzymatic hydrolysis. The possibly novel GSLs were proposed to be mono-, di- and trihydroxyl derivatives of phenethyl GSL. The positions of the OH groups were tentatively proposed, based on IR, formation of ITC versus OAT and from biosynthetic arguments, to be at the *o* or *o*+*p* positions, the latter with and without a 2-hydroxyl group on the aliphatic part. Some MS fragmentation interpretation seems peculiar (lactones formed after loss of two carbonyls and H_2O). Methoxy-hydroxybenzyl GSLs were not discussed as possible structures. Lack of NMR prevented definite conclusions. Finally, "128" listed in Agerbirk and Olsen (2012) was discontinued for reasons discussed elsewhere (Agerbirk and Olsen, 2013). Briefly, the spectral data did not entirely match the suggested structure and a related publication was retracted.

Two sulfate conjugates of GSLs were reported from the gut of a crucifer-feeding insect (Opitz et al., 2011): benzyl GSL 3′-*O*-sulfate and phenylethyl GSL 3′-*O*-sulfate. These structures are by definition natural GSLs. But they were only characterized by elemental composition as determined by MS, and the documentation is insufficient for inclusion in the list so far. Furthermore, our list is limited to plant-biosynthesized GSLs. Nevertheless, the authors proved the ability of the insect to form

3′-*O*-sulfate derivatives of dGSLs, so it is likely that these structures exist as natural GSLs.

A claimed but not documented novel GSL, "4-sinapoyloxybutyl GSL" was studied by Kliebenstein et al. (2007) and the missing evidence reviewed by Agerbirk and Olsen (2012) (page 34). Later on, Lee et al. (2012) studied and revised the biosynthesis of these GSLs, and also mentioned the existence of "4-sinapoyloxybutyl GSL" referring to LC-MS results not shown and peaks in HPLC-DAD chromatograms (at 330 nm) of dGSLs. The latter report supports the existence of this GSL first claimed by Kliebenstein et al. (2007). It is hoped that documentation will be published in order for the claimed compound to be included in the list of documented GSLs.

A good critical review of the analytical chemistry of GSLs (Clarke, 2010), also contained a brief introduction listing no less than 194 "reported" GSLs from plants, as well as a further 180 completely hypothetical structures in an accompanying table. However, critical examination (Agerbirk and Olsen, 2012) showed that this diversity overview is unreliable and not backed up by cited literature. Generally, the structural diversity listed by Clarke (2010) is not a critical review but simply recapitulates previous "reviews" and reports that were also not critical. This is evident from the lack of reference to primary literature for each claimed GSL.

A subsequent MS-based claim (Bianco et al., 2012) of "glycinylglucocapparin" (R-group $CH_2CH(NH_2)COOH$) wrongly cited Clarke (2010) for this GSL being "putative", and perhaps for this reason did not take potential isomers into account (e.g. a hydroxyamide). In the same way, mercaptomethyl GSL was claimed from comparison to MS of methyl GSL and reference to "putative" GSLs listed by Clarke (2010), although the relevant list concerned hypothetic structures. Isomers (methylsulfanyl as well as various isoGSLs, Section 5.1.7.) were not considered, the expectable oxidized dimer (analogous to **134**, Fig. 15H) was not discussed or reported, and the expected ITC not tested for. Two further derivatives were claimed without consideration of isomers or relevant control experiments: "disulfanylmethyl GSL" (R = CH_2-S-SH) and "trisulfanylmethyl GSL" (R = CH_2-S-S-SH). NMR, synthesis and/or identification of expected myrosinase products is required for confirmation of all those claims. If confirmed, "mercaptomethyl GSL" might be the first Cys-derived GSL. Hence, experiments with isotopically labeled precursors would also be an obvious test of the structural hypothesis.

Desulfo-derivatives of disubstituted phenethyl GSLs were isolated from *B. vulgaris* by HPLC and analyzed by UV, MS/MS and ^{1}H NMR (Agerbirk and Olsen, 2012; Agerbirk et al., 2015a). They were each identified as 3/4-hydroxymethoxy derivatives of co-occurring GSLs (**40*R*** and **105**, Fig. 15E), but because of lack of sensitivity in attempted 2D NMR it could not be determined whether the *O*-methyl was at position 3 or 4. MS/MS could not distinguish the two possibilities either. Hence, two novel GSLs provisionally named **x1** (Agerbirk and Olsen, 2012) and **x2** (Agerbirk et al., 2015a) were definitely at hand, but no single exact structure could be proposed (Fig. 17). Stereochemistry of **x1** at the 2-position is tentatively inferred from the dominance of *R*-hydroxylation in the plant source. For **x1**, an isoGSL could be ruled out after demonstrated conversion to the expected product type by myrosinase (Agerbirk and Olsen, 2012).

In a review (Bell and Wagstaff, 2014), several misunderstood GSL structures were propagated, only two examples are detailed here. First, hypothetic 4-phenylbutyl GSL was listed as existing based on D'Antuono et al. (2008); although that report was obviously wrong (t_R of the peak identified as 4-phenylbutyl dGSL was less than t_R of the twice lower homolog **105** in reversed phase HPLC). Second, the review wrongly listed three suggested isobars (**[78]**, **[33]** and **[6]**, Fig. 15G, H, and I) as documented in the investigated species, although the source (Pasini et al., 2012) had merely concluded that an unidentified peak could be one of the three listed candidates. Interpretation of the review (Bell and Wagstaff, 2014) was furthermore complicated by contradictions between names and drawn structures and other features.

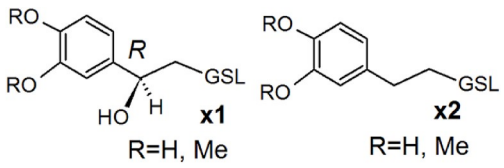


Fig. 17. Two novel glucosinolates demonstrated by NMR and MS, but without conclusion of a single structure since the position of the methyl group is undetermined in each case.

When considering trace-level apparent GSLs reported in papers discussed below, it should be remembered that our knowledge of potential isomerism of the constant part of natural GSLs is virtually zero. Does GSL biosynthesis occasionally make "errors", resulting in "isoGSLs"? (Section 5.1.7.). Such isomers would by definition not be GSLs (Section 1.1.). If so, these side products could be metabolized by oxidation, conjugation etc., resulting in waste products or functional non-GSL metabolites that would appear like GSLs in MS. Recent reports of multiple apparent GSL isomers (Section 5.1.7.) can only be explained if some isoGSLs occur in plants. These findings underline the need for comprehensive structural analysis before even tentatively accepting new GSL structures.

Multiple unprecedented hypothetical GSLs were tentatively suggested from apparently very sensitive HPLC-high resolution MS/MS analysis of *Rorippa indica* (L.) Hiern. (variable leaf yellowcress) (Lin et al., 2014). The detection of many apparent GSLs not contained in the currently accepted list is of interest, suggesting that a myriad of minor GSLs or isoGSLs may exist in this plant. However, the data did not at all allow identification, and some tentative identification did not take possible isomers into account. For example, apparent hexosides were interpreted as glucosides without discussion.

Based on initial MS/MS analysis of authentic GSLs acylated on the glucose residue, *B. vulgaris* seeds were investigated using HPLC-MS, and a range of additional apparent acylated GSLs at very low levels were discovered (Bianco et al., 2014). Detailed analysis of fragments suggested that the additional trace constituents represented the same GSL moieties as in the known 6′-isoferuloyl derivatives **129–132** (Fig. 15F), albeit esterified with other carboxylic acids, probably including coumaric, sinapic and dimethoxycinnamic acids or isomers. Despite extensive interpretation of high-resolution masses, the authors concluded that distinction of the many possible isomers was not possible from MS fragmentation alone.

Numerous GSLs or isomers from various vegetables were claimed from HPLC-MS/MS data processed by a computer program ("GLS-

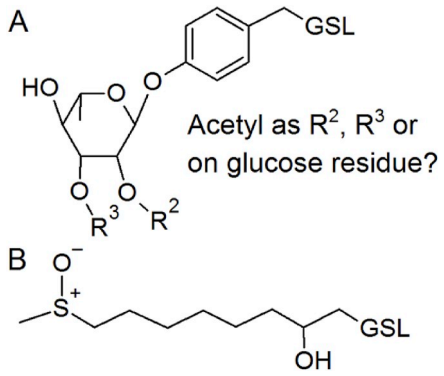


Fig. 18. Some proposed glucosinolates (GSLs) in need of more evidence. (A) Two GSLs suggested from MS to be isomers of **[3]**, but without general NMR evidence or published MS/MS evidence for the position of the acetyl groups at the rhamnose residue, as opposed to the glucose residue or another isomer. (B) 2-Hydroxy-8-(methylsulfinyl)octyl GSL suggested from MS/MS evidence but without NMR evidence.

Finder") (Sun et al., 2016). These inconclusive identifications are further discussed in Section 5.1.7.

What appeared to be a novel GSL, "2-hydroxy-8-(methylsulfinyl) octyl GSL" (Fig. 18B), was reported by Olsen et al. (2016) from detailed interpretation of MS2 data and t_R of a trace peak (Table 1, entry No. "150"). However, NMR was not included and an isoGSL not considered. If a GSL is at hand, formation of an OAT after myrosinase hydrolysis is expected and ought to be confirmed.

Numerous GSLs, including putative structures not included in Table 1, were claimed from analysis not backed up by authentic references of various processed, mixed plant products of industrial origin (Shi et al., 2017). The plant species origins of the individual analytes were not reported, and any presence in gently treated plant samples was not investigated. Hence, the existence in intact plants of the analyzed compounds is uncertain, as are the claimed identities. Finally, isomer distinction was not discussed. The inconclusive nature of MS^n data for GSL identification is further discussed in Section 5.1.

Numerous claims were based on HPLC-MS data without any consideration of t_R or possible isoGSLs or comparison with relevant authentic standards (Capriotti et al., 2018), as further discussed in section 5.1.7.

Two novel GSLs appear to await final evidence; they have been reported from the tropical tree *M. oleifera* that is dominated by the rhamnosylated GSL **110** (Fig. 15F). While the acetyl derivative **[3]** with the acetyl group at the 4′-position of the rhamnose residue is already known and tentatively characterized, the remaining two possibilities (acetyl groups at 2′- and 3′-positions) would appear to exist, according to papers by two independent groups (Maldini et al., 2014; Förster et al., 2015) (Fig. 18A). However, neither group has clearly communicated MS/MS results proving the position of the acetyl groups of the remaining isomers to be on the rhamnose residue (as opposed to the glucose residue), although both groups clearly imply these positions. One paper (Maldini et al., 2014) directly mentions MS/MS results as proving the position of the acetyl group to be on the rhamnose residue, but do not present the results. Acceptance as "partially characterized" of the claimed 4-(2′-*O*-acetyl-α-L-rhamnopyranosyloxy) benzyl GSL and 4-(3′-*O*-acetyl-α-L-rhamnopyranosyloxy)benzyl GSL should await publication of MS/MS data or other data unequivocally documenting the site of substitution to be on the rhamnose residue. Förster et al. (2015) also reported that these derivatives were poorly compatible with the desulfation procedure often employed for GSL analysis (Section 5.1.4.).

It was initially highly exciting to read the first report of variation of the glycosidic part in the side chain of **110** isolated from the tropical horseradish tree (genus *Moringa*). However, the claimed structure, "4-(α-L-glucopyranosyloxy)benzyl GSL" (Fahey et al., 2018), is in contrast to the presented NMR data. In essence, the paper reported an additional GSL in *Moringa* with a mass 16 Da higher than **110**, and suggested the inferred additional OH group to be located in the 6′-position of the rhamnoside unit of **110**. It was additionally suggested in a figure that such hydroxylation of an L-rhamnoside would make it an L-glucoside, ignoring the configuration at C2. The following is a re-evaluation of 12 pages of crude scans of ambiguously hand-annotated spectra, with a contradictory brief summary and without detailed analysis or tabulation of the data.

Comparison to published spectra of **110** and to the synthetic isomer 4-(β-D-glucopyranosyloxy)benzyl GSL (Gueyrard et al., 2010, supplementary figure in Müller et al., 2015) supported our re-evaluation. Three of the pages depict apparently identical ^{1}H NMR spectra, except for an apparent calibration difference of the chemical shifts. In all cases, a prominent methyl group signal (as in the rhamnoside unit in **110**) is present (doublet variously at *ca.* 1.1 and *ca.* 1.2 ppm and integrating to 3H); suggesting that the analyzed sample is a GSL with a rhamnoside moiety (this methyl group would of course be missing in the claimed 6-hydroxy-rhamnoside/"glucoside"). Assuming that the reported elemental composition is correct, we are left to search for another position for the hydroxyl. In contrast to the claimed structure with a *p*-di-substituted benzene ring and hence four aromatic protons, the published spectrum shows only three aromatic protons, apparently a singlet and two doublets with coupling supported by COSY. Hence, the spectrum clearly suggests a GSL with a tri-substituted benzene ring. A candidate identity would be a hydroxy derivative of **110** but with the hydroxyl group situated on the phenyl ring! However, because of the unclear communication, this interpretation is tentative. We conclude that the claim by Fahey et al. (2018) was in contrast to the data, and that critical re-evaluation is hindered by the unclear presentation of data. Had the data supported a hydroxyl in the 6-position of a glycoside, α- and β-linkage (Gueyrard et al., 2010) and the configurations at all other chiral centers in the glycoside moiety would still need consideration, but were not discussed by the authors either.

In the same paper, a specific acetyl derivative was also claimed without presenting any spectroscopic data but only the *m/z* value, although the slightly unclear text (page 3) leaves the incorrect impression that NMR data for both claimed novel GSLs are reported as supplementary data. Hence that claim is ignored here.

3. Syntheses of glucosinolates

3.1. General approach of chemical synthesis

Purification of individual GSLs from plants can be tedious in many cases. Therefore, a chemical synthetic approach has emerged as a more general and efficient way to access pure natural or artificial GSLs.

From the chemist's viewpoint, a generally accepted synthetic scheme has been used for the elaboration of GSL structures (Rollin and Tatibouët, 2011). The key reaction involves the 1,3-addition of 1-thio-β-D-glucopyranose on a labile nitrile oxide (Fig. 19), which has to be generated *in situ* from a hydroximoyl precursor (Benn, 1964a; Cassel et al., 1998; Cerniauskaite et al., 2011). Subsequent sulfation of the obtained glucosyl thiohydroximate followed by deprotection of the carbohydrate moiety affords the desired GSL. Since the pioneering synthesis of **11** by Ettlinger and Lundeen (1957), more than 20 chemical syntheses of naturally occurring GSLs have been described. All structural classes of GSLs are represented in the literature:

- Alkyl GSLs: **[16]** (Keller et al., 1984), **51** (Benn, 1964b), **56** (Benn and Meakin, 1965), **61** (Benn and Yelland, 1967), **[102]** (Lim et al., 2018)
- Alkyl GSLs bearing an oxygenated function: **[26]** (Cerniauskaite et al., 2011)

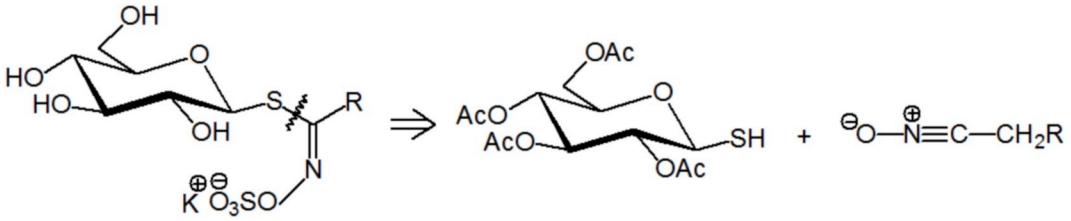


Fig. 19. Retrosynthetic scheme for glucosinolate synthesis.

- Alkyl GSLs bearing a thiofunction: **64** (Iori et al., 1999; Vo et al., 2013), **83** (Yamazoe et al., 2009), **84** (Mavratzotis et al., 1996, 2018), **87**, **[89]**, **94** and **95** (Mavratzotis et al., 2018)
- Alkenyl GSLs, optionally hydroxylated: **12** (Kjær and Jensen, 1968), **24*R*** (Jensen and Kjær, 1971; MacLeod and Rossiter, 1983), **24*S*** (Jensen and Kjær, 1971), **107** (Benn and Ettlinger, 1965; Abramski and Chmielewski, 1996)
- Benzyl GSLs, including side chain *O*-glycosides: **11** (Ettlinger and Lundeen, 1957; Benn, 1963; Vastenhout et al., 2014; Lim et al., 2018), **23** and **46** (Benn, 1965), **110** (Gueyrard et al. 2000, 2010)
- Homobenzyl GSLs: **105** (Benn, 1964c; Gil and MacLeod, 1980; Lim et al., 2018)
- Indol-3-ylmethyl GSLs: **28** (Teranishi and Masayasu, 2016), **43** (Viaud and Rollin, 1990; Cassel et al., 1998; Vo et al., 2014), **47** (Vo et al., 2014), **48** (Viaud et al., 1992; Vo et al., 2014).

3.2. Examples of applications

GSLs bearing a particularly sensitive aglycon could profitably be obtained by chemical synthesis. This is the case for indole GSLs which are to some extent prone to oxidation (Viaud and Rollin, 1990; Viaud et al., 1992; Cassel et al., 1998). In other respects, chemical synthesis can give access to GSLs present in only small amounts or complex mixtures in plants, such as ω-(methylsulfanyl)alkyl GSLs (Mavratzotis et al., 2018). The availability in reasonable amounts of such synthetic GSLs can facilitate further biological studies. For instance, a study of the enzymatic degradation of indol-3-ylmethyl GSL (**43**) by myrosinase was performed on a synthetic sample (Latxague et al., 1991). However, similar studies of indole GSL biochemistry (e.g. Hanley and Parsley, 1990; Bonnesen et al., 1999) have been carried out using GSLs isolated from plant sources such as broccoli (Agerbirk et al., 1998) or *A. thaliana* (Kim and Jander, 2007).

In contrast, isotope-labeled GSLs are highly useful for the investigation of biosynthetic and metabolic pathways and reaction mechanisms, and can only be obtained by synthesis. For example, the enzymatic, chemical, and thermal degradation of synthetic tritium-labeled **43** was qualitatively and quantitatively studied by LC-MS, GC-MS, and radioactivity counting (Chevolleau et al., 1993, 1997). In another example investigating GSL biosynthetic pathways, 3,4-T_2-but-3-enyl GSL (3,4-T_2-**12**) was synthetized and fed to oilseed rape (*Brassica napus* L.). This tritium-labeled **12** was incorporated into the 2-hydroxy derivative **24*R*** and thus shown to be its biosynthetic precursor (Rossiter and James, 1990). In yet another case, allyl GSL (**107**) was known to form either the thiocyanate or ITC product upon myrosinase-catalyzed hydrolysis, depending on the presence or absence of thiocyanate-forming protein in field pennycress (*Thlaspi arvense* L.). Using **107** deuterated at the side chain double bond, the formation of the thiocyanate was confirmed to result from a rearrangement involving connection of the thiohydroximic S to the terminal C, in parallel with the Lossen rearrangement delivering the ITC (Rossiter et al., 2007) (Section 4.3.).

In other biosynthetic studies, turnip (*Brassica rapa* L.) roots were UV-irradiated, fed with synthetic [4,5,6,7-D_4]**43**, and incubated for 4 days. D_4-Indole-3-carboxaldehyde and D_4-methyl indole-3-carboxylate were isolated, indicating that in such conditions the metabolism of the parent indole GSL **43** was unrelated with phytoalexin biosynthesis (Pedras et al., 2002). However, when synthetic [2,2,4,5,6,7-D_6]**43** was fed to UV irradiated leaves of salt cress (*Thellungiella salsuginea* = *Eutrema salsugineum* (Pall.) Al-Shehbaz & Warwick), the labeled GSL was metabolized to wasalexin A, wasalexin B, biswasalexin A1, 1-methoxy-, and 4-methoxy**43** (= **47** and **48**) (Pedras et al., 2010). More recently, synthetic [2,2,4,5,6,7-D_6]**43** was fed to UV-elicited rutabaga (*Brassica napus* L.) roots and a maximum of three deuterium atoms from hexadeuterated **43** were incorporated into the phytoalexins rapalexin A, isocyalexin A, and isalexin. Additionally, [2,2,4,5,6,7-D_6]**43** was incorporated intact into cyclobrassinin, spirobrassinin, **47** and **48**, demonstrating that **43** is the biosynthetic precursor of all these natural products (Pedras and Yaya, 2013).

A different set of experiments concerned phenethyl GSL (**105**) and its *m*-hydroxy derivative **148** (Fig. 15E), and their possible biosynthetic connection to newly discovered non-indole phytoalexins (Section 4.4.). In support of this hypothesis, [2,3,4,5,6-D_5]**105** was fed to elicited leaves of watercress (*Nasturtium officinale* R. Br.) and was metabolized into [2,3,5,6-D_4]nasturlexin B and [2,3,5,6-D_4]tridentatol C. In addition, synthetic [2,4,6-D_3,^{15}N]**148** was fed to elicited leaves of upland cress (*Barbarea verna* (Mill.) Asch) and metabolized into [2,4,6-D_3,^{15}N]-3-hydroxyphenylethyl ITC, [2,4,6-D_3,^{15}N]dihydronasturlexin D, and [2,4,6-D_3,^{15}N]nasturlexin D. However, when the same labeled GSL was fed to non-elicited leaves, it was only metabolized into [2,4,6-D_3,^{15}N]-3-hydroxyphenylethyl ITC (Pedras and To, 2018). Finally, in the same investigation, synthetic [2,3,4,5,6,7,8-D_7]-(*E*)-styryl GSL was not incorporated into any metabolite when fed to *B. verna* leaves, indicating that it is not likely a biosynthetic precursor of nasturlexins C or D (Pedras and To, 2018).

An application for the determination of GSLs by enzyme-linked immunosorbent assay (ELISA) was developed via synthesizing the *p*-carboxyphenyl analogue of phenyl GSL (Fig. 20) (Bromet et al., 1993). Further coupling of this hapten to bovine serum albumin allowed production of anti-GSLs polyclonal antibodies.

Other examples relate to the substrate specificity of myrosinase. Eight glyco-analogs were synthesized by replacing the glucose unit of **43** by another sugar moiety. Those were probed as substrates for myrosinase (Gardrat et al., 1993; Palmieri et al., 1993). In addition, several *α*-anomeric counterparts of GSLs (benzyl GSL (**11**), **105** and **43**) were produced by synthesis (Blanc-Muesser et al., 1990) and those substrates were shown to be reluctant to myrosinase hydrolysis (Joseph, 1993). Moreover, several deoxy analogs of **11**, 2-fluoro-2-deoxy**11**, and several deoxy analogs of **43** were synthesized to understand the mechanism of hydrolytic cleavage of GSLs by myrosinase (Streicher et al., 1995; Iori et al., 1996; Cottaz et al., 1996, 1997) (Fig. 21). With a view to estimating the role of the anionic site during the GSL hydrolytic process by myrosinase, a phosphate analog of **11** was elaborated (Lazar and Rollin, 1994). A synthetic *C*-analogue of **11** in which the anomeric sulfur atom is replaced by a methylene group has been reported (Aucagne et al., 2000). Subsequent inhibition experiments demonstrated that the *C*-benzyl GSL was not recognized by myrosinase. This is the sole example of a glycosylhydrolase that does not recognize the *C*-analogue of its natural substrate (Aucagne et al., 2000). Additionally, 5a-thia**11** and 5a-thia**43** were synthesized (Joseph and Rollin, 1993) (Fig. 21). Synthetic 5a-carba**11** was further tested as a substrate for myrosinase and was shown to be the first example of a non-hydrolysable GSL analogue to inhibit myrosinase (Lefoix et al., 2002).

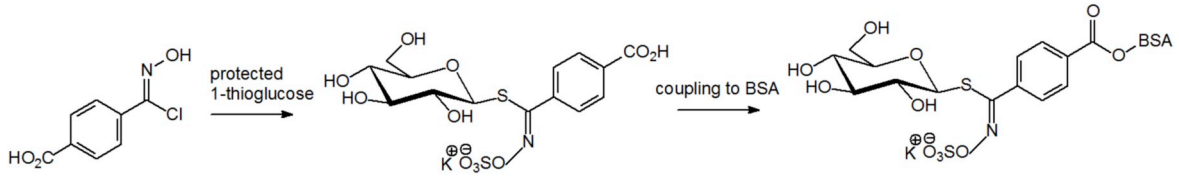


Fig. 20. Synthesis of a glucosinolate epitope coupled to bovine serum albumin (BSA), intended for elicitation of an immune response.

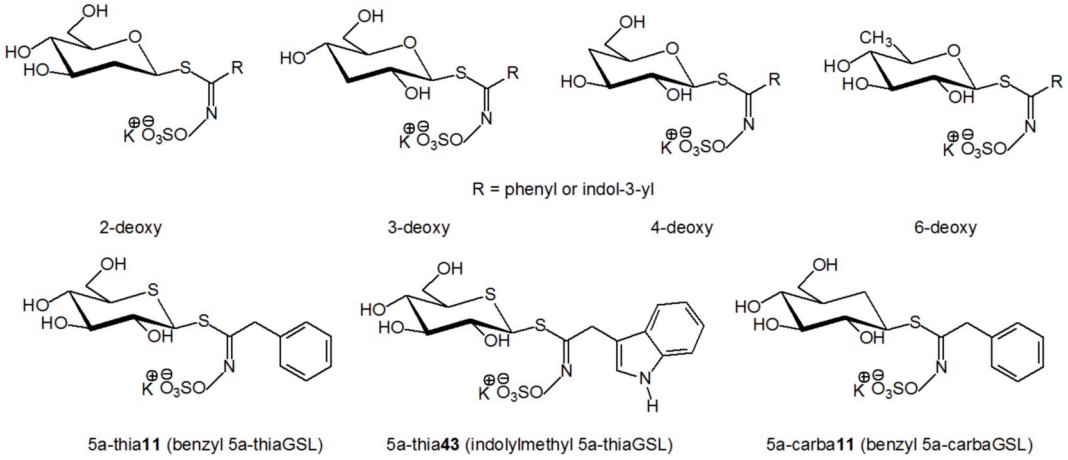


Fig. 21. Selected examples of glucosinolate analogs synthesized for studying the interaction of natural glucosinolates with myrosinase.

4. Catabolism of glucosinolates by plant enzymes

In this review, we confine the discussion to catabolism and turnover of GSL in the plant, as opposed to catabolism in animals after GSL intake (e.g. Traka, 2016; Jeschke et al., 2016; Soundararajan and Kim, 2018; Hanschen et al., 2019) and during domestic or industrial processing (Hanschen et al., 2014; Nugrahedi et al., 2017). However, we do include catabolism in damaged plant cells, as it happens upon physical damage to GSL-containing plants.

4.1. Classical myrosinases

Myrosinases are defined by catalytic function, not by sequence. They are thioglucoside glucohydrolases (EC 3.2.1.147) that can cleave a GSL to produce an unstable aglucone under glucose release (Fig. 22). Myrosinase activity has, as far as we know, been detected in all GSL-containing plants investigated for this activity to date. Most known sequences (but see Section 4.2.) belong to the glycoside hydrolase family 1 (Xu et al., 2004) and are called the classical myrosinases. In rape (*B. napus*) and white mustard (*S. alba*), twenty or more (Xu et al., 2004) isoforms of myrosinase have been described (Thangstad et al., 1993; Rask et al., 2000). Different myrosinase isoforms in these and other plant species differ in their solubility and their distribution among plant organs and tissues (Lenman et al., 1993; Rask et al., 2000; Eriksson et al., 2001; Travers-Martin et al., 2008; Andersson et al., 2009; Loebers et al., 2014). The crystallized myrosinase from *S. alba* seeds is a homodimer stabilized by a Zn^{2+} ion (Burmeister et al., 1997), and the soluble myrosinases in *B. napus* and *S. alba* were reported to be highly glycosylated homodimeric proteins with an apparent molecular weight of 130–150 kDa of which carbohydrates can account for up to 20% (Rask et al., 2000). Gel-filtration experiments suggested larger molecular complexes in seeds of some *Brassica* species (Bellostas et al., 2008), and the molecular weight of wasabi (*Wasabia japonica* = *Eutrema japonicum* (Miq.) Koidz.) myrosinase was found to be 580 kDa, corresponding to 12 subunits (Ohtsuru and Kawatani, 1979). Little is known about the impact of quaternary myrosinase structure on protein stability or enzyme kinetics. Investigations aimed at answering biological aspects are even more challenging, because the defensive capacity of the GSL-myrosinase system depends on myrosinase activity in chemical environments like animal guts. The *S. alba* myrosinase MA1 was recently shown to resist digestive proteolysis by larvae of the generalist lepidopteran cotton leaf-worm (*Spodoptera littoralis* Boisd.) (Vassão et al., 2018), underlining the pronounced stability of myrosinases, which may be critical for their function.

In plant tissues, myrosinases are stored spatially separated from their GSL substrates, which was proposed as the mechanism that prevents *in planta* GSL hydrolysis; this mechanism was metaphorically termed the “mustard oil bomb” (Matile, 1980; Lüthy and Matile, 1984). A number of studies using antibodies have localized myrosinases to idioblastic “myrosin cells” (Werker and Vaughan, 1974; Thangstad et al., 1990; Husebye et al., 2002; Ueda et al., 2006), after the term had been coined by Guignard (1890). GSL and myrosinases are, however, not always stored in separate cells. Compartmentalization can also be achieved through localization of both components in different cell parts, reviewed by Kissen et al. (2009). The complex spatial organization of the GSL-myrosinase system suggests that the evolution of plant defense systems required novel anatomical and subcellular structures (Kliebenstein, 2013).

Myrosinases catalyze cleavage of the thioglucosidic bond in a two-step reaction (Burmeister et al., 1997, 2000; reviewed by Wittstock et al., 2016). First, a nucleophilic attack at the anomeric carbon by a Glu residue in the enzyme liberates the aglucone. After this first step, the glucose moiety is covalently bound to the active site Glu residue in myrosinase. Secondly, the free enzyme is restored by hydrolysis of the Glu-glucose bond, facilitated by activation of a water molecule required for glucose release. This second and rate-limiting step is facilitated by ascorbate, a cofactor of classical myrosinases (Ettlinger et al., 1961; Ohtsuru and Hata, 1979; Shikita et al., 1999; Burmeister et al., 2000). Other glycosidases typically contain a second Glu residue able to protonate the aglycone, thereby making it a better leaving group. Due to the weak base nature of the GSL aglucone (Section 1.1.), such protonation is not required for GSL hydrolysis. In fact, a common feature of classical myrosinases is the replacement of a catalytic Glu (E) residue present in the other members of the glycoside hydrolase family 1 within the peptide TFNEP motif. The corresponding sequence at the active site of classical myrosinases is characterized by a Gln (Q) residue within a TI/LNQL/P motif. A secondary function of the second Glu residue in classical glycosidases - activation of H_2O - is taken over by ascorbate, as a general base-catalyst (Burmeister et al., 2000).

Turnover numbers of purified myrosinases are relatively low (0.26–287 s^{-1}, according to the BRENDA database (www.brenda-enzymes.org) and dependent on plant species, GSL structure, pH, temperature and other parameters. With apparent K_M values for their GSL substrates between 10 μM and 6 mM, myrosinases reach rather low *in vitro* efficiencies (k_{cat}/K_M) of 13–85 $mM^{-1}\,s^{-1}$ (BRENDA database), which may explain why they tend to be highly abundant. While most myrosinases catalyze the hydrolysis of a wide variety of GSLs with comparable efficiencies (Durham and Poulton, 1990; James and Rossiter, 1991; Chen and Halkier, 1999; Barth and Jander, 2006), others display a distinct substrate specificity (MacLeod and Rossiter, 1986; Bernardi et al., 2003; Loebers et al., 2014). In some but not all cases, broad and narrow substrate specificities reflect the chemical

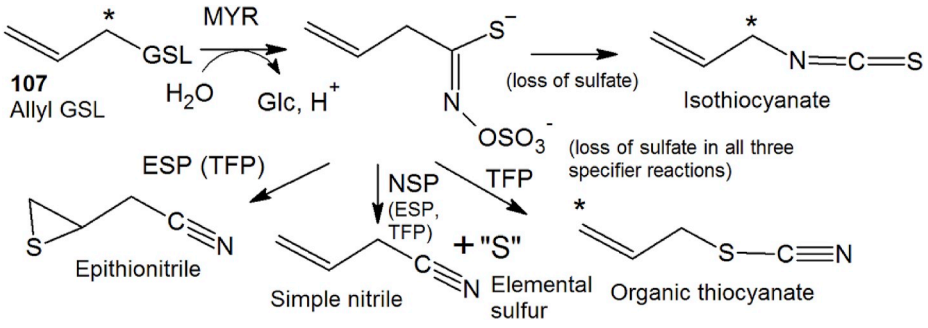


Fig. 22. Diverse products of allyl glucosinolate (GSL) depending on presence or absence of specifier proteins. The asterisks over the GSL, the isothiocyanate and the thiocyanate summarize the results of labeling studies (Benn, 1977; Rossiter et al., 2007) as previously detailed (Agerbirk and Olsen, 2012). All GSLs can form isothiocyanates, although some may be unstable, and nitriles. Only aliphatic GSLs with a terminal unsaturation can form epithionitriles. Only allyl GSL and two other GSLs (formally able to form a resonance-stabilized cation) can form organic thiocyanates. Some specifier proteins have other activities than those they are named from, as indicated in brackets. MYR, myrosinase; ESP, epithiospecifier protein; NSP, nitrile specifier protein; TFP, thiocyanate-forming protein.

diversity of the coinciding GSLs. For example, the myrosinase isolated from seeds of abyssinian kale (*Crambe abyssinica* = *Crambe hispanica* subsp. *abyssinica* (Hochst. Ex R.E.Fr) Prina) shows a strong preference for **24*S*** (Fig. 15I), the major GSL structure in those seeds (Bernardi et al., 2003). In contrast, myrosinase MY1 from horseradish (*Armoracia rusticana* P. Gaertn., B. Mey. & Scherb.), shows a broad substrate specificity and an equally broad expression pattern, while MY2 expression is limited to young roots and favors the GSL substrate predominant in this tissue (Loebers et al., 2014).

4.2. Atypical myrosinases in plants

Myrosinase activity is not restricted to classical myrosinases. In *A. thaliana*, two related *β*-glucosidases have been demonstrated to possess thioglucosidase activity despite the presence of a particular Glu residue as acid/base catalyst in their active sites (which is usual for glycosidases but not found in classical myrosinases). The peroxisomal *β*-glucosidase PENETRATION2 (PEN2, BGLU26, At2g44490) has been identified as myrosinase based on its ability to confer resistance to non-adapted filamentous plant pathogens (Lipka et al., 2005). Recombinant PEN2 hydrolyzes the Trp-derived GSLs **43** and **48** (Fig. 5A) *in vitro* resulting in the accumulation of breakdown products of these GSLs *in planta*, together indicating a preference for these substrates (Bednarek et al., 2009; Clay et al., 2009). For resistance against the grass powdery mildew (*Blumeria graminis* (DC.) Speer), PEN2-mediated catabolism of **48** is especially critical (Bednarek et al., 2009; Clay et al., 2009). Unlike the classical myrosinases, PEN2 initiates GSL catabolism in a tissue disruption-independent manner, providing an example of a cell-autonomous GSL-myrosinase system. In response to pathogen infection, PEN2 is directly recruited to the pathogen contact site (Fuchs et al., 2016), where it potentially releases high local concentrations of biologically active GSL hydrolysis products.

Another member of the same subclade of *β*-glucosidases, PYK10 (At3g09260, BGLU23), has been associated with the mutualistic endophytic interaction between *A. thaliana* and the root colonizing fungus *Piriformospora indica* Sav. Verma, Aj. Varma, Rexer, G. Kost & P. Franken (*Serendipita indica* (Sav. Verma, Aj. Varma, Rexer, G. Kost & P. Franken) M. Wei, Waller, A. Zuccaro & Selosse) (Sherameti et al., 2008). Plants lacking PYK10 do not benefit from the growth-promoting effect of *P. indica* colonization and instead suffer from excessive fungal growth in the root tissue (Sherameti et al., 2008). PYK10 is a glycoprotein showing myrosinase activity towards the Trp-derived GSL **43** as well as towards a coumarin *O*-glucoside (scopolin) *in vitro* (Matsushima et al., 2003; Ahn et al., 2010; Nakano et al., 2017). PYK10 is mostly expressed in seedlings and roots of adult plants, and is selectively accumulated in endoplasmic reticulum (ER) bodies, where it represents the major protein component (Matsushima et al., 2003, 2004). In rosette leaves, wound-inducible ER bodies specifically accumulate BGLU18 (At1g52400) and are transcriptionally associated with GSL biosynthesis (Ogasawara et al., 2009; Nakano et al., 2017). Yet, the *in vivo* substrates of BGLU18 remain to be identified. Likewise, future investigations will show whether other relatives of PEN2 and PYK10 possess myrosinase activity. Interestingly, inducible ER bodies have so far only been found in plants of the Brassicales (Nakano et al., 2017), raising the question whether these organelles co-evolved with atypical myrosinases.

4.3. Non-isothiocyanate products from myrosinase-catalyzed hydrolysis

As described in Section 1.1., myrosinase-catalyzed hydrolysis of the thioglucosidic bond in GSLs leads to the formation of sulfated thiohydroximates, which spontaneously rearrange into ITCs (Fig. 22). In contrast, for the formation of most alternative hydrolysis products, specifier proteins are required in addition to myrosinase (Wittstock and Burow, 2007, 2010) (although ferrous ion and low pH can also lead to nitrile formation *in vitro*). Depending on the GSL side chain structure and the properties of the specifier protein present, epithionitriles, simple nitriles and/or organic thiocyanates are formed (Fig. 22). Specifier proteins have no hydrolytic activity on GSLs themselves. Based on their product profiles, they are named epithiospecifier proteins (ESPs), nitrile specifier proteins (NSPs), and thiocyanate-forming proteins (TFPs). However, the grouping is not straight forward, as some specifiers have several activities (Kuchernig et al., 2012; reviewed by Wittstock et al., 2016).

Only one GSL, allyl GSL (**107**), can form all three kinds of alternative products (Fig. 22). Epithionitrile formation occurs only from a small number of GSL precursors with an aliphatic unsaturated side chain, as the sulfur released from the thioglucosidic bond is transferred to a terminal double bond to form a thiirane moiety. The separation from myrosinase of the protein critical for epithionitrile formation from **24*S*** in *C. hispanica* subsp. *abyssinica*, designated ESP, was first reported by Tookey (1973). To date, ESPs have been identified in broccoli, (*Brassica oleracea* ssp. *italica*) (Matusheski et al., 2006), *A. thaliana* (Lambrix et al., 2001), and a number of cabbages and other Brassicaceae (e.g. Kuchernig et al., 2012; Hanschen and Schreiner, 2017; Kolodziejski et al., 2019). Similar to epithionitrile formation, the formation of thiocyanates has only been reported in a relatively small number of plant species and is furthermore restricted to only a few parent GSL structures, namely allyl GSL (**107**), 4-(methylsulfanyl)butyl GSL (**84**), and benzyl GSL (**11**) (Gmelin, 1959; Walker and Gray, 1970; Schlüter and Gmelin, 1972; Burow et al., 2007; Kuchernig et al., 2011). This narrow range of substrates for thiocyanate formation has intrigued researchers for decades (for an excellent introduction, see Benn (1977)), and would seem to have a mechanistic basis (ability to form a stable cation after loss of SCN^- from the ITC) (Benn, 1977). Indeed, labeling studies have shown a complex rearrangement in the formation of allyl thiocyanate from **107** (Rossiter et al., 2007) (Fig. 22), and a mechanism based on the crystal structure of a TFP has recently been

proposed (Backenköhler et al., 2018). Finally, the formation of simple nitriles by myrosinase and NSP appears to be independent of the GSL side chain and represents the ancestral specifier protein activity (Burow et al., 2009; Kissen and Bones, 2009; Kuchernig et al., 2012). The formation of non-ITC products is common in Brassicaceae, but not in other families of the Brassicales, and accordingly, no specifier proteins have been identified outside the Brassicaceae (Kuchernig et al., 2012).

Structurally, the specifier proteins are related. ESPs and TFPs typically have a molecular weight of 30–40 kDa, while many characterized NSPs are larger due to one or two "jacalin"-related lectin domains at their N-terminus (Nagano et al., 2008; Burow et al., 2009; Kuchernig et al., 2012). Across the three different types, specifier proteins share 50–80% amino acid sequence identity (Kuchernig et al., 2012). The C-terminal part of all specifier proteins was predicted to be characterized by a series of β-sheets known as Kelch-motifs (Burow et al., 2006; Matusheski et al., 2006). Recent elucidation of the crystal structures of ESP and NSP1 from *A. thaliana* and TFP from *T. arvense* demonstrated that all three proteins adopt a six-bladed β–propeller structure (Gumz et al., 2015; Zhang et al., 2016b, 2017). Molecular modelling combined with mutational analysis of the *T. arvense* TFP further revealed iron-binding sites essential for TFP activity (Brandt et al., 2014; Gumz et al., 2015). The presence of iron (suggested to be ferrous ion) in specifier proteins (Backenköhler et al., 2018) further supports a critical role of this element in their catalytic mechanism. This issue has been under debate since the characterization of the first specifier proteins (Tookey, 1973; Foo et al., 2000; Lambrix et al., 2001; Burow et al., 2006; Wittstock and Burow, 2007; Kissen and Bones, 2009).

4.4. Additional reactions

GSLs are hydrolyzed upon tissue disruption or fungal infection, which results in release of the immediate products of GSL hydrolysis into the plant's headspace or apoplast or the mouth or gut of a herbivore. Depending on side chain structure, some ITCs undergo spontaneous chemical cyclization reactions into OATs or other heterocycles or loose the ITC functional group as thiocyanate ion (Section 1.1.3.; Table 1). However, GSL hydrolysis occurs also in the absence of tissue damage and biotic attackers. During the transition from seed to seedling, the total GSL content decreases (Brown et al., 2003; McGregor, 1988) and young seedlings can utilize exogenous GSLs as a sulfur source under sulfur deficiency (Zhang et al., 2011). A number of recent studies strongly suggest that GSL turnover plays a critical role in fine tuning of GSL profiles and plant development (Katz et al., 2015a, 2015b; Francisco et al., 2016a, 2016b; Malinovsky et al., 2017; Urbancsok et al., 2017, 2018), but the pathways for GSL turnover in intact plant tissues are generally not yet well understood.

A well-studied case is cell-autonomous GSL degradation as mediated by PEN2 (Section 4.2.), repeatedly reviewed (Pastorczyk and Bednarek, 2016; Wittstock et al., 2016; Czerniawski and Bednarek, 2018). This GSL activation leads to accumulation of indol-3-ylmethylamine and raphanusamic acid which are produced from the glutathione conjugate of indol-3-ylmethyl ITC derived from **43** (Bednarek et al., 2009). In *A. thaliana*, the glutathione-*S*-transferase GSTU13 (AT1G27130) has been shown to contribute to GSH conjugation of indol-3-ylmethyl ITC (Piślewska-Bednarek et al., 2018) (Fig. 23).

In addition, an *N*-substituted formamide derivative carrying a 4-β-glucopyranosyloxyindol-3-yl substituent and named "4-*O*-β-D-glucosyl-indol-3-yl formamide" using un-orthodox nomenclature (*N*-(4-β-D-glucopyranosyloxyindol-3-yl)formamide is a preferable name for the depicted structure) has been shown to accumulate in response to *B. graminis* infection in a PEN2-dependent manner (Lu et al., 2015). However, the depicted structure (lacking a methyl group next to the indole ring) would seem to require the action of a chain-shortening step (Pedras et al., 2016) for being derived from a conventional indole GSL.

Several recent reports have also shed light on the biosynthesis of phytoalexins from GSL-derived ITCs, a huge, rapidly developing scientific field of its own (Pedras et al., 2011) that can only be superficially reviewed here. Also in this case, the initial GSL turnover to ITCs must be catalyzed in intact cells, and the responsible myrosinase is unknown, although candidate genes have been proposed (Klein and Sattely, 2017). A simple example is brassinin, shown conclusively to be a product of the parent Trp-derived GSL **43** (Fig. 24) (Pedras and Yaya, 2013). In a fascinating recent paper, it is suggested with model compounds how this transformation can happen in a way that recapitulates the core GSL biosynthesis, but ending with a methyl transferase to form the sulfur-containing group of brassinin (Klein and Sattely, 2017). Two cytochromes P450 convert brassinin to further phytoalexins, spirobrassinol and cyclobrassinin (Klein and Sattely, 2015) (Fig. 24). Biosynthetic cyclization to the 4-position of the indole ring has also been described, leading to cyclonasturlexin, described as "the most intriguing indole phytoalexin isolated to date" (Pedras and To, 2016) (Fig. 24). Another highly surprising crucifer phytoalexin was an aromatic isothiocyanate (rapalexin A) that did not correspond to any proteinogenic amino acid precursor (Pedras et al., 2007). In two incisive papers (Pedras and Yaya, 2013; Pedras et al., 2016), Trp and **43** were nevertheless demonstrated to be biosynthetic precursors of rapalexin A (Fig. 24). A novel chain shortening mechanism was demonstrated, including the novel non-protein amino acid 4-methoxyindol-3-ylglycine and the suggested (but unstable) novel GSL **[147]**. Not all crucifer indole phytoalexins are GSL derived (e.g. Glawischnig, 2007).

Recently, "crucifer non-indole phytoalexins" (Pedras and To, 2015; Pedras et al., 2015) from wintercress (*Barbarea* spp.) and watercress (*Nasturtium* spp.) were also demonstrated to be GSL-derived using isotopic labeling (Section 3.2.). In this case, the precursor GSL was phenethyl GSL, **105**, via phenethyl ITC (Fig. 25).

Also nitriles are subject to further reactions. Enzymatic conversion of nitriles to amides and carboxylic acids in crucifers depends on bifunctional "nitrilases" that are functionally both nitrile hydratases (E.C. 4.2.1.84) and nitrilases (E.C. 3.5.5.1), equivalent to a single reaction with H_2O or two consecutive reactions with H_2O (Fig. 26). Despite variable main products, molecular phylogenies are coherent, and classification according to protein phylogeny is preferable to classification according to main reaction catalyzed (Janowitz et al., 2009). In *A. thaliana*, four enzymes are known, *At*NIT1-4. For the *At*NIT4 enzyme, involved in ethylene metabolism, both the amide and acid are major products (Piotrowski et al., 2001; Piotrowski and Volmer, 2006). The

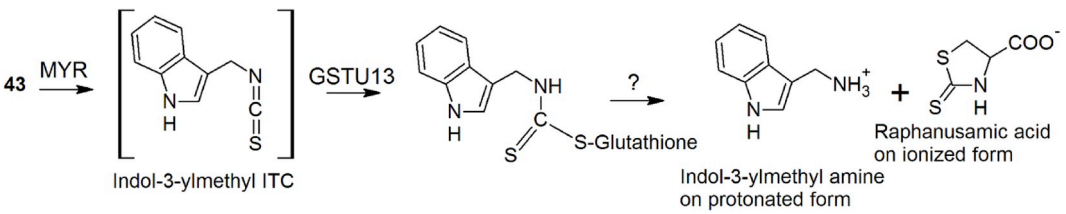


Fig. 23. Participation of glutathione and a glutathione-*S*-transferase in forming a GSL-derived amine as a product of glucosinolate metabolism in intact cells, important for plant immunity. Question marks above some arrows signify steps that are demonstrated *in vivo* but for which specific enzymes are not yet known. Reactions are unbalanced. Amine and acid products are illustrated as the corresponding ammonium and carboxylate ions, as they would mainly exist at physiological pH. MYR, myrosinase.

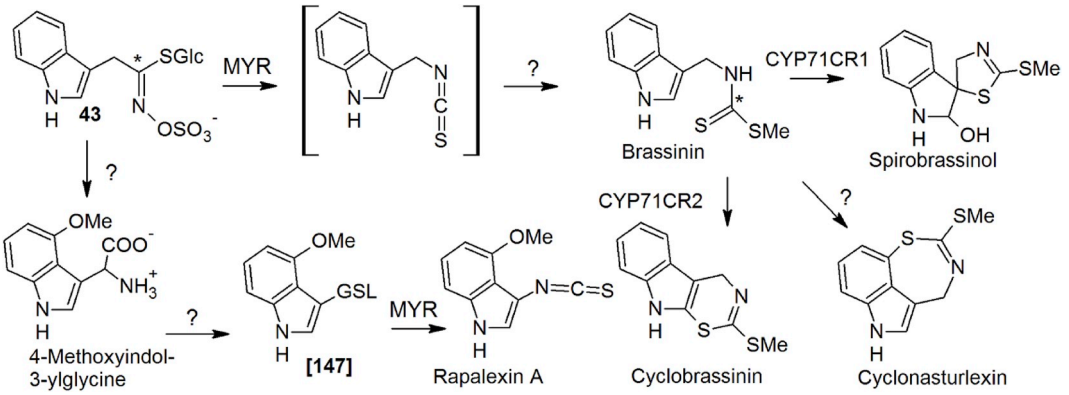


Fig. 24. Examples of glucosinolate-derived indole phytoalexins and biosynthetic connections. Asterisks indicate a selected example of a labeling study of the brassinin biosynthesis (Pedras and Yaya, 2013). Question marks above some arrows signify steps that are demonstrated *in vivo* but for which specific enzymes are not yet known. MYR, myrosinase.

nitrilases *At*NIT1-3 are closely related and part of a crucifer-specific nitrilase family ("NIT1 group") suggested to be involved in GSL biochemistry mainly based on phylogeny and substrate specificity (Janowitz et al., 2009). The latter enzymes also produce a mixture of amides and carboxylic acids *in vitro* (Osswald et al., 2002), but have usually been assayed based on the ammonia product (Bestwick et al., 1993; Vorwerk et al., 2001), meaning that little is known concerning amide formation. However, assays in crude extracts of *Sinapis* spp. showed amide formation to be strictly proportional to carboxylic acid formation. In *S. arvensis* L. (charlock mustard) the amide was the dominant product, while in *S. alba* the carboxylic acid product dominated by far (Agerbirk et al., 2008). The responsible cDNAs were cloned and expressed, and the nitrilases confirmed to be of the NIT1 group (*Sal*NIT1c, *Sar*NIT1c) with the same ratio of amide to carboxylic acid products as found with crude extracts. Experiments based on site-directed mutagenesis showed that change of specific amino acid residues drastically affected the product ratio (Trompetter, 2010). Related nitrilases (*Cr*NIT1a, *Cr*NIT1b) from pink shepherd's-purse (*Capsella rubella* Reut.) were also characterized, including aspects of substrate specificity (Trompetter, 2010; Woodward et al., 2018). The physiological role of the NIT1 subgroup is still not fully understood and could be exerted in intact cells (Kutz et al., 2002; Frisch et al., 2014; Lehmann et al., 2017; Urbancsok et al., 2018), in plant tissue macerates after tissue disruption (as usually assayed) or in insect guts (Agerbirk et al., 2007). Crucifer nitrilases have been reviewed repeatedly (Piotrowski, 2008; Janowitz et al., 2009; Agerbirk and Olsen, 2012; Wittstock et al., 2016).

Additional putative downstream products of GSL turnover were reviewed previously (Agerbirk and Olsen, 2012), and some additional examples are listed here (Fig. 27A–E). Raphanusanin isomers are cyclization products of 4-(methylsulfanyl)but-3-enyl ITC from **83** (Fig. 15H) (Hasegawa et al., 2000; Moehninsi et al., 2010, 2014). This and alternative fates of the ITC from **83** were reviewed by Montaut et al. (2010a). Alliarinoside (Haribal et al., 2001) is a glucosylated nitrile suggested to be biosynthesized from allyl GSL (**107**) via the epithionitrile (Frisch et al., 2015) in garlic mustard, *Alliaria petiolata* (M.Bieb.) Cavara & Grande. In the same plant, the glucosylated amide

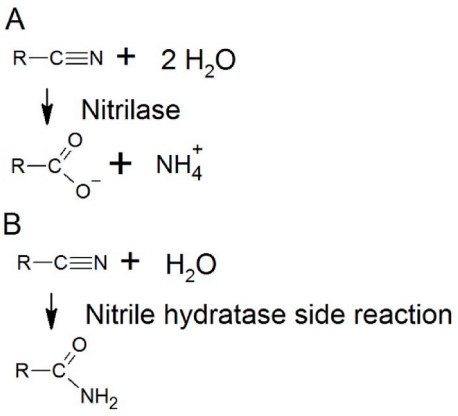


Fig. 26. Mixed reaction catalyzed by "nitrilase" enzymes in crucifers. (A) The strict functional definition of nitrilase activity (E.C. 3.5.5.1). (B) A side reaction catalyzed by crucifer nitrilases to variable degree, probably caused by premature termination of the reaction after the first round of addition of water (Jandhyala et al., 2005), functionally defined as nitrile hydratase activity (E.C. 4.2.1.84).

petiolatamide suggested a complex metabolism of GSLs in intact cells (Frisch et al., 2014). Macamides are suggested to be formed from benzylamines derived from benzyl GSL (**11**, Fig. 15D) and its derivatives in maca, *Lepidium meyenii* Walp., after hydrolysis of ITCs to amines *in vivo* (reviewed by Huang et al., 2018). Even more complex sulfur compounds in maca, thiohydantoins, were also suggested to be GSL derivatives (Huang et al., 2018).

Some atypical products of GSL breakdown after tissue disruption have also been described recently (Fig. 27F–H). Kjaerin is a thiazolidin-2-one formed by fast rearrangement of the corresponding isomeric OAT from **139*R*** (Fig. 15E) (and expected from **139*S*** as well) (Agerbirk and Olsen, 2015). Only the *p*-hydroxy isomer resulted in a thiazolidin-2-one, the *m*-hydroxy isomer GSL **142*R*** (Fig. 15E) formed the usual OAT, exemplifying the importance of specific isomers for reactivity. An

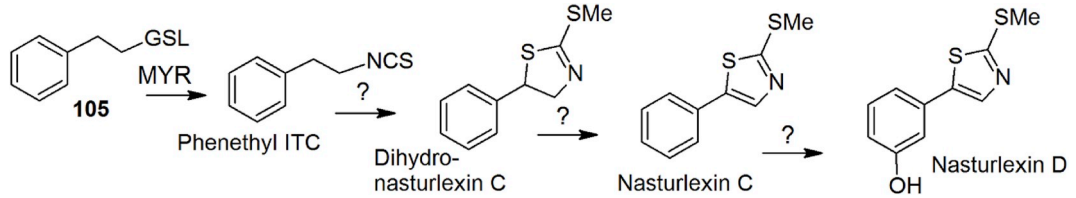


Fig. 25. Examples of crucifer non-indole phytoalexins derived from phenethyl isothiocyanate (Pedras and To, 2018). Question marks above some arrows signify steps that are demonstrated *in vivo* but for which specific enzymes are not yet known. MYR, myrosinase.

aldehyde was apparently formed as a product of **152** (Fig. 15D) (Pagnotta et al., 2017), after deduced loss of thiocyanate ion due to an activating *p*-hydroxyl group (Fig. 8). The cyclic product of **133,** already described in section 1.1.3. (Fig. 8), is caused by an intramolecular reaction of the ITC group with a thiol. (*R*)-Resedine and (*S*)-resedine are chiral oxazolidin-2-ones formed from the corresponding OATs from **40*R*** and **40*S*** (Fig. 15E) in several Brassicales species (Agerbirk et al., 2018; Müller et al., 2018) and blue discoloration in radish roots

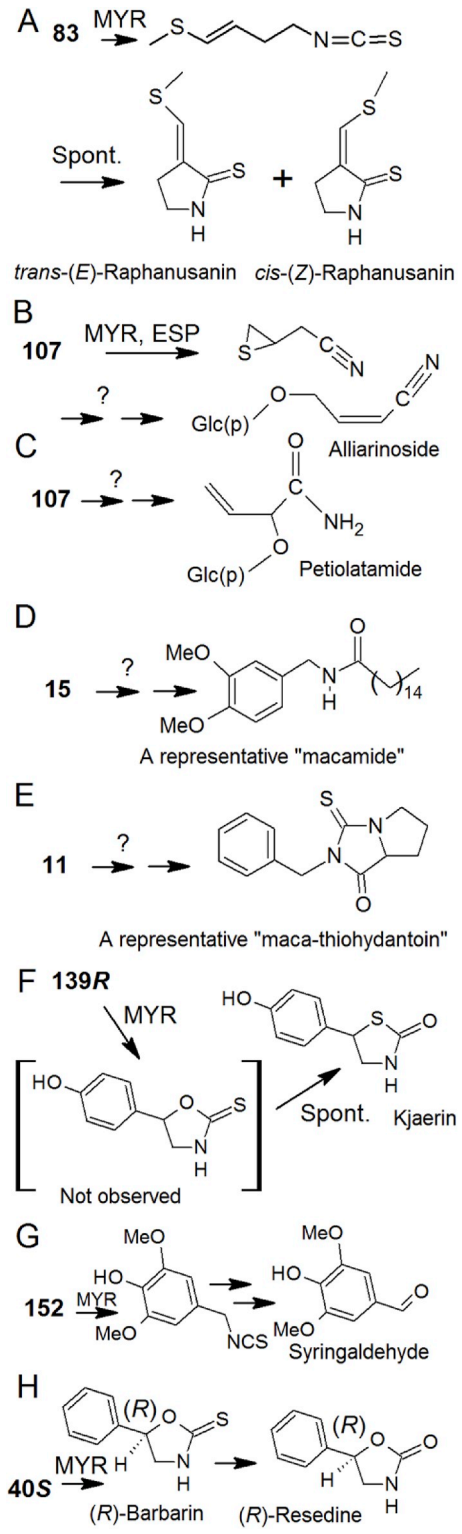


Fig. 27. Suggested downstream products of glucosinolate (GSL) turnover *in planta* in addition to those discussed in a previous review (Agerbirk and Olsen, 2012). (A) Formation of a cyclic growth regulator from **83** via the isothiocyanate (ITC). (B) Suggested formation of an insect feeding deterrent, alliarinoside, from **107** via the epithionitrile. (C) Suggested formation of a glucosylated amide from **107**, possibly via the nitrile and amide. (D): One of many "macamides" in "maca", suggested to be formed from benzyl GSL and derivatives via the ITC and the free amine, followed by amide formation with fatty acids. (E) One of numerous sulfur compounds in "maca" suggested to be formed in complex ways from benzyl GSL and derivatives. (F) Activation of the heterocyclic ring at position 4 in an oxazolidine-2-thione resulted in isomerization, in this case to kjaerin, 5-phenylthiazolidin-2-one. Spelling of thiazolidin-2-one is correct here (without "e" before hyphen because the following letter is a vowel) and was wrong in the original report. (G) Although the corresponding alcohol was expected from autolysis of *Lepidium* seeds containing the *p*-hydroxybenzylic **152**, the main product was the aldehyde, apparently due to non-enzymatic oxidation. (H) Substitution of the S with an O by a heat sensitive factor, "OATase", detected in several Brassicales species. Single enantiomers of barbarin gave single enantiomers of resedine (Müller et al., 2018). MYR, myrosinase.

consisted of unidentified oxidation products of the indole GSL **28** (Fig. 15C) and were influenced by peroxidase (Teranishi and Masayasu, 2016; Teranishi et al., 2016).

In some cases, it is uncertain whether unusual products are limited to the food matrix or also found in native crushed plants. Two examples are the formation in mustard of the problematic trace food constituent bisphenol F (bis-(4-hydroxyphenyl)methane) from *p*-hydroxybenzyl GSL (**23**, Fig. 15D) via 4-hydroxybenzyl ITC (Zoller et al., 2016) and the cyclization of the epithionitrile from allyl GSL (**107**) into 2-aminothiophene (Hanschen et al., 2018a).

In conclusion, GSL degradation products are much more diverse than imagined some decades ago. In particular, repeated demonstrations of GSLs as biosynthetic intermediates in intact cells have convincingly shown that an understanding of GSLs as passive components of a "bomb" is too narrow an understanding of their biochemistry. In many cases, newly discovered GSL products depend on specific structural features of the GSL side chains, underlining the biological significance of side chain structure and the importance of analytical isomer distinction.

5. Hyphenated techniques for glucosinolate identification and quantification: potentials, requirements and pitfalls

Before the availability of GC and HPLC, GSL identification relied on comparison with authentic standards of GSLs (or ITCs after controlled myrosinase breakdown of GSLs) in at least two chromatographic or electrophoretic systems. Quantification was difficult and at best approximate. With the advent of GC-FID and later HPLC-UV, and optimization of the conditions, quantitative analysis of well-understood sample types such as rapeseed, mustard, radish and cabbage became possible. This is still the case, and HPLC-UV is reliable for analysis of very well-characterized species and sample types (e.g. commercial oilseed rape and *Brassica* leaf crops, mustard seeds, *A. thaliana* samples from well-known genotypes) (e.g. Reichelt et al., 2002; Brown et al., 2003; Wathelet et al., 2004; Moreira-Rodriguez et al., 2017; Possenti et al., 2017; Hanschen et al., 2018b). In these cases, the retention time (t_R) is sufficient evidence, in combination with the previous knowledge of the GSLs that can be expected, and the added specificity of the derivatization to dGSLs or a preceding group separation of intact GSLs on ion exchange cartridges. Capillary electrophoresis of intact GSLs (Michaelsen et al., 1992) and dGSLs (Bjergegaard et al., 1995) is equivalent.

However, the lack of suitability of many GSL products for GC was and is a problem and the inability to separate all GSLs and dGSLs at any single HPLC condition is another. Essentially, t_R is not a sufficient

analytical parameter for identification when all known and yet unknown GSLs are potential candidates (Kiddle et al., 2001). Hyphenation of HPLC to diode array UV detectors improved the situation for GSLs containing an aromatic group ("aromatic GSLs", Fig. 10). UV spectra from diode array detectors are a strong supplemental tool in that case. Notably, subtle differences in substitution pattern can lead to clearly distinguishable differences in UV spectra. A number of papers show UV spectra of aromatic dGSLs commonly observed in *Brassica* species etc. (Kiddle et al., 2001; Wathelet et al., 2004; Grosser and van Dam, 2017) or lists UV spectral information of a wider range of dGSLs: **d28**, **d43**, **d47**, **d48**, **d138** (Agerbirk et al., 2001b), benzoylated aliphatics in general (Reichelt et al., 2002), **d23**, **d46**, **d140**, **d143** (Agerbirk et al., 2008), **d129**, **d130**, **d131*S***, **132*R*** (Agerbirk and Olsen, 2011), and **d15**, **d22**, **d114**, **d151**, **d152** (Pagnotta et al., 2017).

Hyphenation of GC or HPLC to MS represented a huge increase in analytical power. Suddenly, identification of known analytes gained much more certainty, and *m/z* values and MS/MS data efficiently narrowed down the number of structural possibilities of unknowns. The widespread availability of MS detectors led to increased quality in GSL analysis in the hands of careful workers using authentic standards, e.g. GC-MS of ITCs (Kjær et al., 1963; Spencer and Daxenbichler, 1980), GC-MS of pertrimethyl-silylated dGSLs (Christensen et al., 1982; Shaw et al., 1989), HPLC-MS of intact GSLs (Fabre et al., 2007; Mohn et al., 2007; Maldini et al., 2012; Montaut et al., 2018), and HPLC-MS of dGSLs (Windsor et al., 2005; Agerbirk et al., 2008, 2014; Kusznierewicz et al., 2013; Olsen et al., 2016).

Reaching certainty in GSL diversity in *A. thaliana* has been a gradual process with many contributions, summarized below with emphasis on the role of different techniques. In a pioneering paper on HPLC-MS of dGSLs from *A. thaliana* (Hogge et al., 1988), the authors did not use authentic dGSL standards for identification, but instead parallel GC-MS analysis compared with the MS data published in literature was used for GSL confirmation. However, Reichelt et al. (2002) provided certainty regarding most of the *A. thaliana* GSLs using NMR analysis of desulfo derivatives, followed up with comprehensive quantitative data by Brown et al. (2003). Further analytical progress was gained using capillary electrophoresis-MS (Bringmann et al., 2005) and using HPLC-MS of intact GSLs (Glauser et al., 2011). In conclusion, reliable HPLC-MS of *A. thaliana* GSLs relied on peak identification using NMR and authentic references, exchange of well-characterized genotypes, and analytical development. The same reliability should be required of any other species analyzed and has been realized in many cases (e.g. Table 1). Notably, each new species considered gains support from the reliable part of the previous literature, especially if references or characterized seeds are made available (Section 5.1.1.).

A less certain case was pioneering method work by Mellon et al. (2002) followed by extensive screening by Bennett et al. (2004), resulting in an inspiring amount of preliminary GSL diversity data for seeds of a large number of species. No use of NMR or authentic standards was documented, but extracts with previously known GSLs from well-known species can be regarded as implicit standards. So data from Bennett et al. (2004) need be interpreted case-by-case for each GSL. Some claims were later confirmed by NMR (e.g. **140**, **143**) (Table 1) while other claims were contradicted as discussed below.

Unfortunately, the temptation to base identification on *m/z* values or MS/MS data alone, without consideration of t_R or use of standards, has also led to an explosion of obviously unreliable GSL data, as discussed separately for HPLC-MS and GC-MS. Sadly, the peer review system combined with editorial control has not been able to avoid this unfortunate development, not even in the case of dedicated and generally respected journals. Hence, great care is needed in interpretation of the current literature (Agerbirk and Olsen, 2012; Olsen et al., 2016) (Section 2.7.). For example, the claimed presence of unbranched GSLs in *Cardamine pratensis* L. (cuckoo flower) based on HPLC-MS (Bennett et al., 2004) could not be confirmed using NMR analysis, while isomeric branched structures were identified (Agerbirk et al., 2010a). In contrast, the precise isomers reported in *Cardamine diphylla* (Michx.) Alph. Wood (toothwort) (Feeny and Rosenberry, 1982) using controlled myrosinase hydrolysis and GC calibrated with authentic ITC standards were indeed confirmed using NMR analysis (Montaut and Bleeker, 2013; Olsen et al., 2016), although additional GSLs were identified in the newer investigations. Indeed, some seemingly archaic papers using authentic references may be more reliable than recent literature not based on authentic standards.

In the following, identification of GSLs directly by HPLC and indirectly by GC-MS of ITCs and other products is discussed separately because of the many individual features.

5.1. HPLC-MS

From the discussion below and a previous review (Agerbirk and Olsen, 2012), the main requirements of reliable HPLC-MS identification of GSLs and dGSLs should conform to standard, traditional natural product chemistry/analytical chemistry practices, and are:

1) For the entire reported data to be reliable, it should be made clear for each reported individual GSL whether identification is conclusive or tentative. Use of authentic standards or reference materials is mandatory for conclusive identification by HPLC-MS. GSL analysis should not be attempted without calibrating the analytical setup with a considerable number of authentic GSL or dGSL standards, obtained as pure compounds or as constituents of well-characterized reference materials.
2) In general, reliable peak identification of known GSLs needs at least two independent analytical characters, of which the retention time (t_R) should be one, and the mass spectrum (preferentially including MS/MS) would be the other. In case of novel or rare GSLs, where an authentic standard is not available, it is still critical to compare the t_R with relevant standards of similar structure, e.g. homologs or corresponding oxidized or non-oxidized compounds, to reach a qualified guess. Known properties of GSL biosynthesis should be taken into account when proposing tentative structures, and still, full identification requires NMR. In no case should GSL identification be attempted from MS data alone, without critical consideration of the t_R.
3) When interpreting *m/z* values, even from high-resolution mass spectrometers, the possibility of isomers should always be critically considered and any uncertainty communicated clearly. When only nominal mass is available, the possibility of isobars should also be considered. Targeted HPLC-MS/MS (in which only preselected peaks are recorded) is an efficient tool for quantification but is not necessarily conclusive identification, depending on the ions selected for monitoring. Targeted analysis must be combined with untargeted analysis (and/or literature analysis) in order to conclude total GSL profiles.
4) The limitations and information from extraction and sample preparation of intact GSLs and dGSLs should be considered while respecting their strengths and weaknesses.

Since these requirements are critical, it follows that any scientific report including GSL identification should specify the origin and type of reference material used, the extraction and sample preparation conditions, and the basis of identification of the actual individual HPLC peaks. In other words, GSL identification should be transparent, allowing the reader to judge specifically the evidence for each reported GSL. Many recent papers describe peak identifications that are obviously unreliable, making the entire data less trustworthy. In contrast, explicit distinction of certain and uncertain peaks is a genuine benefit for the reader, in this way advancing science and giving lasting credit to the authors. Labeling of tentative identifications in tables is simple, e.g. by brackets or question marks.

The mentioned analytical requirements (1–4) are discussed one by

one in the following.

5.1.1. Authentic standards and reference materials

Approximately twenty authentic GSLs are currently commercially available (Liang et al., 2018). An obvious alternative, and often the only source of a given GSL, is seeds or other parts of well-characterized species (e.g. Kiddle et al., 2001; Wathelet et al., 2004; reviewed by Blažević et al., 2017). Simple, direct comparison with a reference species is sometimes used as a structural argument (Mikkelsen and Halkier, 2003), although use of a specific accession already characterized is of course preferable. Certified rapeseed standards with either low, medium or high levels of GSLs are commercially available (Sigma-Aldrich; ERMBC366, ERMBC190, ERMBC367) (Wathelet et al., 1998; Hrnčiřík et al., 1998; Linsinger et al., 2001), and many *A. thaliana* ecotypes are also very well characterized in terms of GSL profile (Kliebenstein et al., 2001b; Brown et al., 2003; Hanschen et al., 2018b). Many other species can be obtained by ordering previously analyzed seed batches from commercial suppliers or seed banks. Simple small-scale GSL isolation (Section 7) from such sources allows anyone to obtain a number of authentic references independent of commercial availability. One author (NA) has deposited seeds of a number of characterized *Arabis*, *Barbarea*, *Planodes* and *Sinapis* species freely available at www.nordgen.org/sesto. They may be used to produce most indole GSLs (Fig. 15C), homoPhe derived GSLs like **40*R***, **40*S*** and numerous rare derivatives (Fig. 15E) as well as many Met-derived GSLs (Agerbirk et al., 2008, 2010b). Likewise, commercially available *C. pratensis* (Section 7.1.) is a good source of several branched aliphatic GSLs including the novel **58**, **141** and **149** (Fig. 15B) (Agerbirk et al., 2010a; Olsen et al., 2016). Finally, a number of GSL samples can be obtained by chemical synthesis (Section 3).

5.1.2. Retention time

Lists or graphs of typical t_R values, based on authentic standards, can be found in several papers (intact GSLs; e.g. Millán et al., 2009; Glauser et al., 2011; Maldini et al., 2012; Ares et al., 2014; Crocoll et al., 2017) (dGSLs; e.g. Kiddle et al., 2001; Agerbirk et al., 2001b; Brown et al., 2003; Wathelet et al., 2004; Kusznierewicz et al., 2013; Bhandari et al., 2015; Olsen et al., 2016; Crocoll et al., 2017; Pagnotta et al., 2017; Grosser and van Dam, 2017), allowing some general rules to be set up for reversed-phase HPLC. Added hydrophobic moieties result in increased t_R in a logical, predictable way. That is, members of a homologous series will elute in order of increasing number of methylene groups, with approximately regular spacing when a simple linear gradient is used. Added hydroxyl groups confer the opposite trend, and the effect of a hydroxyl group often reduces t_R approximately as much as a methylene group increases t_R. Substituents in "aromatic GSLs" (Fig. 10) follow the same logical patterns. Methylsulfanylalkyl GSLs and dGSLs are rather hydrophobic (high t_R) reflecting the low polarity of the C–S bond. In contrast, the corresponding methylsulfinylalkyl and methylsulfonylalkyl GSLs have much reduced t_R. The latter two exhibit similar t_R, illustrating a small polarity difference between these two functional groups. Recently, hydrophilic interaction liquid chromatography (HILIC) has been applied, resulting in values of t_R that are approximately a reversal of the order observed in reversed-phase chromatography (Liang et al., 2018).

If an exact authentic standard is not available, a preliminary identification from comparison with homologs is a generally respected argument due to the semi-predictable nature of the t_R, and is part of the scant evidence for some long chain GSLs like Met derived **[74]**, **[85]**, and **[89]** (Fig. 15G).

5.1.3. Isomers and isobars

Isobars are compounds that have the same nominal mass without being isomers, e.g. from the fact that a phenyl group has the same nominal mass (77) as a methylsulfinylmethyl group. This source of uncertainty should be eliminated by NMR, UV or high resolution MS for reliable structural elucidation. For any molecular formula, a surprising number of isomers is usually possible. Isomers often display slightly different t_R, but when many isomers are possible, co-elution of some is the rule rather than the exception. For example, careful combination of HPLC using several stationary phases and differential MS/MS fragmentation was needed to distinguish isomers of hydroxyphenethyl GSLs and derivatives (Agerbirk et al., 2015a). In the absence of authentic standards, NMR is needed for distinction (Section 2.2.) unless all possible isomers have been previously studied and shown to exhibit different MS/MS and/or t_R. As discussed below (Section 5.1.7.), it cannot even be assumed that all detected isomers are GSLs at all.

For these reasons, targeted analysis by the "MRM" approach (Section 5.1.5.) does not necessarily represent conclusive identification; it is quite likely that isomers will show a similar t_R and share some MS fragments. This is definitely the case if general GSL or dGSL fragments form the basis for the quantification and selectivity (e.g. *m/z* 97, $[HSO_4]^-$, from intact GSLs (Liang et al., 2018) or glucose, thioglucose, anhydroglucose or their sulfate derivatives). When isomers show differences in fragmentation, the MRM approach could be designed to take advantage of this for conclusive isomer distinction. However, such methods have to our knowledge not yet been published. Obviously, targeted analysis only, which ignores all GSLs not taken into account by the preselected targeting, is not a reliable tool for determining the entire GSL profile of general samples.

It has often been assumed but never proven that MS of GSLs and dGSLs has predictive value if interpretation fits a given structure. However, in addition to fragmentation, extensive rearrangement takes place (e.g. Bianco et al., 2012; Bianco et al., 2014) complicating interpretation. Systematic study of rules of fragmentation of GSLs and dGSLs, using authentic standards, is in its infancy, but some studies are available (for GSLs, see Bialecki et al., 2010; Bianco et al. (2017), and for dGSLs, see e.g. Agerbirk et al. (2014, 2015); Olsen et al. (2016); Pagnotta et al. (2017)). Briefly, at least two side chain fragmentation types are well established: loss of $H(SO)CH_3$ from ω-(methylsulfinyl) alkyl GSLs and dGSLs, and loss of aldehydes after an aldol-cleavage-like fragmentation of *β*-hydroxyalkyl GSLs and dGSLs (but not in cases where the hydroxyl group is terminal, e.g. **57*R*** (Figs. 15A) and **30** (Fig. 15B)). Alkyl side chain branching cannot be distinguished by MS of GSLs and dGSLs, in contrast to the situation in GC-MS of myrosinase products (Section 5.2.2.).

5.1.4. Qualitative aspects of sample preparation

Some GSLs constitute specific analytical problems. 4-Hydroxyindol-3-ylmethyl GSL (**28**, Fig. 15C), which is the dominant GSL in modern rapeseed, is less stable than most GSLs during storage due to sensitivity to oxidation, but stabilization using an added thiol was reported (Sang and Truscott, 1984). It is a well-known observation that high pH or drying under an airstream can lead to complete disappearance of **28**, associated with a bluish color. Recently, 1-hydroxyindol-3-ylmethyl GSL (**[154]**) was reported to be even more unstable (Pfalz et al., 2016).

The use of enzymatic desulfation (Fig. 28) is very popular, and for good reasons (Section 2.1.). However, some GSLs cannot be determined with this strategy. Two rare GSLs with a negatively charged side chain are known, **112** (Fig. 15C) and **[155]** (Fig. 15A). Since their desulfo derivatives will not be eluted from the anion exchange material used for desulfation, these GSLs cannot be determined by the usual desulfo-procedure. Comparing analysis methods of the acetylated GSL **[3]** and isomers (Figs. 15E and 18), it was found that acetyl groups are lost during desulfation (Förster et al., 2015), meaning that acetyl derivatives of **110** cannot be determined in this way. Likewise, partial hydrolysis of the rhamnoside bond in **110** (Fig. 15F) was observed during desulfation, resulting in *p*-hydroxybenzyl GSL (**23**) as an artifact among dGSL derivatives (Förster et al., 2015; Müller et al., 2015). A suitable method based on intact GSLs, avoiding hydrolysis of **110**, was devised (Förster et al., 2015). The above GSLs are known or suspected to be scarce (Bennett et al., 2004), so this desulfation problem is only

troublesome for a small number of species, but underlines the relevance of including methods based on intact GSLs whenever a new plant species is studied.

As reviewed previously (Agerbirk and Olsen, 2012), the desulfo derivative of a GSL occurring in radish, 4-(methylsulfinyl)but-3-enyl GSL (**63**, Fig. 15H) and is less stable than most dGSLs (Iori et al., 2008; De Nicola et al., 2013a), thus leading to analytical difficulties (Wathelet et al., 2004). Indeed, lower relative peak areas were confirmed (Doheny-Adams et al., 2017) but the GSL was correctly identified at all sample preparation conditions. The dimeric oxidized product (**134**) obtained from 4-mercaptobutyl GSL (**133**, Fig. 15H) was in one study observed only after desulfation, suggesting it to be an artifact of desulfation (Doheny-Adams et al., 2017), in contrast to a classic, elegant investigation suggesting the dimer to be a native GSL (Cataldi et al., 2007).

Although there are problems associated with desulfation in GSL analysis, there are also advantages. Simple interpretation due to simpler chromatograms, well-established quantification and simple scale up for isolation and NMR are general advantages. In addition, structure-specific fragmentation of sodium adducts of dGSLs has been documented, in many cases allowing isomer distinction and confirmation of structural elements (Olsen et al., 2016 and references cited therein).

Sampling may be critical for the GSLs to be detected. It is well established that leaves, roots and seeds often show qualitative differences (reviewed by van Dam et al., 2009), due to transport and tissue-specific biosynthesis (Nour-Eldin et al., 2012; Andersen et al., 2013), but the extent of variation within plant parts is less well-known so far. Since vegetative plant parts are heterogeneous with respect to GSL distribution and biosynthesis (Schroff et al., 2008; Sønderby et al., 2010b; Madsen et al., 2014; Nintemann et al., 2018), sampling entire leaves (rather than leaf disks) is needed for representative results. The same is likely relevant for various parts of cabbages etc. Hence, careful planning and sample reduction is needed for representative sampling of vegetative plant parts.

5.1.5. Quantitative aspects of sample preparation and peak detection

The two major principles of GSL analysis by HPLC, based on intact GSLs or desulfated derivatives (Section 2), have recently been illustrated with numerous detailed practical considerations in two clearly written publications (Crocoll et al., 2017; Grosser and van Dam, 2017). The latter has focused on a simple method and is accompanied by a video, but unfortunately specifies a wrong type of anion exchange material for binding the GSLs, probably due to a typing error. Some earlier references also provided carefully explained step-by-step instructions (Sørensen, 1990; Wathelet et al., 2004).

Common for both approaches is extraction. Avoidance of enzymatic breakdown of GSLs is of paramount importance for reliable extraction. The conventional wisdom in the GSL field recommended brief extraction in hot aqueous alcohol (e.g. Harborne, 1973) to inactivate the enzymes. During standardization of methods for analysis of oilseed rape, a hot extraction (75 °C for 10 min) was selected, apparently without further optimization (Buchner, 1987a), and this was eventually fixed in a European Union standard and the equivalent ISO 9167-1 method widely used in GSL analysis (Anon, 1990; Anon, 1992). This procedure was originally developed for seed analysis, but also analysis of vegetative parts has been found reliable in terms of reasonable agreement with levels of breakdown products after autolysis (e.g. De Nicola et al., 2013a; Klopsch et al., 2018; Pagnotta et al., 2017; Hanschen et al., 2018b). One author (NA) has for two decades been using a shorter hot extraction (3x boiling extraction in 70% aq. MeOH, each for 1.0 min), and also found this shorter hot extraction reliable in terms of reproducibility, linearity, reasonable agreement with levels of individual GSL breakdown products and very low levels of GSL breakdown products in immediately extracted samples (Agerbirk et al., 2001a, 2007, 2014; Agerbirk and Olsen, 2015). These results suggest that it may be possible to reduce the 10 min duration of hot extraction in the EU/ISO 9167-1 method, and in this way combine myrosinase inactivation with reduced thermal loss of GSLs.

In the *A. thaliana* community, it was discovered that even cold extraction of *A. thaliana* leaves can be performed without significant GSL hydrolysis as long as 80% aq. MeOH is used as solvent, and this protocol has become close to standard for analysis of *A. thaliana* (but some workers also use the ISO 9167-1 extraction, see Kissen et al., 2016; Hanschen et al., 2018b). This cold extraction has not been published with extensive controls and discussion included. Extension of this cold extraction to other plants was questioned, citing the scarce literature critically testing cold extraction and showing insufficiency for mustard seeds (Agerbirk and Olsen, 2012). A later systematic investigation considered vegetative parts of four plant species (Doheny-Adams et al., 2017). The samples were subjected to either hot or cold extraction, and similar or marginally higher extracted levels from the cold extraction were generally found. However, with one sample type (radish stems) the yields using the cold extraction were much reduced (Doheny-Adams et al., 2017), indicating that cold methanolic extraction of GSLs can be inadequate. A microwave alternative has been described (Ares et al., 2014). In the case of cauliflower, a careful, systematic evaluation showed room temperature extraction (in 70% aq. MeOH) to give more GSL-like but not fully identified metabolites than conventional hot extraction (Capriotti et al., 2018), suggesting that room temperature extraction may be an advantage in those cases where myrosinase activity is efficiently inhibited at room temperature. In conclusion, GSL extraction of poorly known sample types should use the conventional brief extraction in hot aq. MeOH, and later deviations from this protocol should be systematically optimized and documented.

The analysis based on dGSL derivatives was initially developed for quantitative analysis of GSLs in rapeseed, and subjected to stringent optimization, standardization and inter-laboratory calibration (Sang and Truscott, 1984; Wathelet, 1987; Anon, 1990; Anon, 1992; Linsinger et al., 2001). General relative response factors for UV detection were determined based on intact GSL standards (Buchner, 1987b), and later generally confirmed and extended for *A. thaliana* GSLs (Brown et al., 2003) and also extended to some additional structures (Wathelet et al., 2004). Hence, the analysis based on dGSL derivatives is quantitatively and qualitatively reliable for the set of GSLs occurring in rapeseed and *A. thaliana*, when adhering to the original conditions. Among potential sources of error in GSL desulfation is desulfation kinetics and differential elution from DEAE-Sephadex anion exchange columns (e.g., Buchner, 1987a, 1987b, Quinsac and Ribaillier, 1987; Wathelet et al., 2004; Doheny-Adams et al., 2017). In particular, indole GSLs with a free NH group (such as **28**, **43** and **48** as well as their desulfo derivatives) and GSLs acylated with phenolic acids (Fig. 15C + F) are eluted later from DEAE-Sephadex columns than usual internal standards and need extended elution for correct analysis (Sang and Truscott, 1984; Buchner, 1987a, 1987b; Agerbirk et al., 2001b; Agerbirk and Olsen, 2011). The essential control to be carried out in every lab is elution of one extra mL of dGSLs from the ion exchange column during a *B. napus* seed GSL analysis. If that final mL is not free from **d28**, the elution

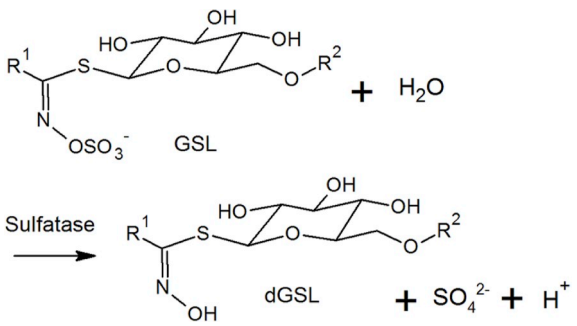


Fig. 28. The desulfation reaction catalyzed by sulfatase and used for derivatization in glucosinolate analysis.

volume is too small.

The crude enzyme preparation from the Burgundy snail (*Helix pomatia* L., Sigma S9626) currently used for desulfation is not pure, and the consensus is that it needs some purification before analytical use, although the reasons are poorly documented. Unpublished results by NA and CEO showed around 20% lower yields of the Phe-derived **11** and **23** (Fig. 15D) relative to an internal standard of allyl GSL (**107**) when using crude enzyme compared to further purified enzyme, suggesting contamination of the crude enzyme with a glucosidase activity with some specificity for phenylalkyl GSLs (perhaps a side reaction of contaminating β-glucuronidase as specified in Sigma-Aldrich product information sheet). This effect of sulfatase purification seems to comply with the oral and sparsely written tradition in the field (Sang and Truscott, 1984). These experiments were based on initial measurement of relative levels of GSLs using ^{1}H NMR of mixtures of pure intact GSLs (**11** with **107** and **23** with **107**) (Agerbirk et al., 2006, 2007), and confirmed the established relative response factors for **11** (Buchner, 1987b) and **23** (Brown et al., 2003). No comparison of the various sulfatase purification protocols has been published, but the simple procedure by Graser et al. (2001) is often adopted. A more elaborate purification procedure was described by Heaney and Fenwick (1980) and Kiddle et al. (2001). Marketing of catalytically pure sulfatase would be most welcome.

While HPLC-UV quantification depends on molar absorptivity that can to some degree be predicted from structure, quantification by HPLC-MS depends on ionization efficiency, which is sensitive to experimental conditions and co-eluting peaks. Some authors have therefore resorted to parallel HPLC-UV for quantification (Agerbirk et al., 2008; Lee et al., 2013; Olsen et al., 2016; Moreira-Rodriguez et al., 2017) or reported semi-quantitative results (Bennett et al., 2004; Windsor et al., 2005). Quantification by MS can be done reliably only when pure authentic standards or reference materials are available, and is often based on subsequent fragmentations in MS/MS ("MRM-monitoring") (Maldini et al., 2012; Ares et al., 2014; Crocoll et al., 2017; Franco et al., 2016; Jørgensen et al., 2017; Liang et al., 2018). However, comparing with the well-established quantification of many GSLs using HPLC-UV could be a future shortcut to quantitative HPLC-MS (Millán et al., 2009; Lee et al., 2013; Ares et al., 2014). In quantitative HPLC-MS analysis, the ultimate method is measurement against added isotopically labeled analogs of the analytes (Clarke, 2010). This procedure takes into account all ionization interferences. Current GSL analysis by MS is far from reaching that goal due to the non-availability of labeled reference compounds. However, advances in GSL chemical synthesis (Section 3.) would in principle open up opportunities for this currently costly approach.

Hence, HPLC-MS quantification depends on availability of pure standards and is currently far from perfect (Clarke, 2010). In HPLC-UV, this is also the case for unusual and rare GSLs (e.g. Berhow et al., 2013), as is the case in many other fields of secondary metabolite analysis. However, even in cases where absolute quantification is uncertain, the influence of various factors (e.g. genes, growth conditions, antagonists) on relative GSL levels can still be reliably tested as long as linearity is ensured. Indeed, this is a common situation in biological GSL research.

5.1.6. Aspects of a single peak identification by HPLC-MS in recent literature

Unfortunately, there are numerous examples of obviously wrong peak identifications in the literature, occurring due to the missing adherence to general natural product chemistry/analytical chemistry practices (Agerbirk and Olsen, 2012). Many examples of inconclusive GSL identification by HPLC-MS were given in Section 2.7. Due to their illustrative features, a few additional cases will be discussed in the following text.

In an otherwise good paper (Gioia et al., 2018), one peak assigned to *n*-pentyl dGSL (d**[102]**) was obviously misidentified, since the t_R was lower than for the polar 4-(methylsulfinyl)butyl dGSL (**64**). One of the stereoisomers of isobaric 2-hydroxybut-3-enyl dGSL (**d24**) seems more likely. As results and methods were reported in detail, the reader can make this mental revision easily. In another report, 2–10 Da deviation of *m/z* values from the expected was attributed to extraction conditions (!) (Hall et al., 2014). This is impossible and must be ascribed to a wrong MS calibration or wrong GSL identification. This shows the necessity of analytically qualified reviewers for evaluating papers employing HPLC-MS analyses.

In an interesting method paper on targeted HPLC-MS/MS analysis, Liang et al. (2018) reported appreciable levels of the rare **40*S*** in broccoli, Brussels sprouts, cabbage and rocket salad, and reported this GSL as a minor constituent of most vegetables analyzed, without discussing this surprising finding in relation to the literature. This result would seem to question the validity of identifications based on the targeted analysis using the MRM approach, at least using that particular protocol. The possibility of isomers was not discussed either, although no known GSL has more known isomers than **40*S***: **40*R***, **45**, **46**, **140**, **148** (Fig. 15 D + E). In essence, even results of advanced methods must be evaluated critically.

In assigning tentative structures, it is useful to observe that well-characterized GSL profiles of plants usually exhibit an inherent biosynthetic logic (e.g. Sang et al., 1984b; Daxenbichler et al., 1991; Brown et al., 2003; Windsor et al., 2005; De Nicola et al., 2012; Agerbirk et al., 2015a; Pagnotta et al., 2017, Klopsch et al., 2018). For example, series of homologs often co-occur in the case of aliphatic GSLs, substituted GSLs are often accompanied by non-substituted counterparts, and end-products of long biosynthetic pathways are often accompanied by the known intermediate GSLs (e.g. terminal unsaturation or hydroxylation accompanied by methylsulfinylalkyl GSLs) (Fig. 6). In a study of horseradish (Agneta et al., 2012, 2014), hypothetic "2-methylsulfonyl-oxo-ethyl GSL" was tentatively proposed, without any discussion of the lack of any other sulfonyl, any other oxo-derivative, and any other non-chain elongated Met derivative among the detected GSLs. In the same papers, 4-mercaptobutyl GSL (**133**) was tentatively proposed without considering the possibility of isomeric 3-(methylsulfanyl)propyl GSL (**95**), even though biochemically related 3-(methylsulfinyl)propyl GSL (**73**) was reported from the same plant. By inspecting known species containing these two isomers (Table 1), it is evident that comparison with widely available plants, a vegetable and an ornamental plant, would have resolved the question immediately, increasing the value of the publication.

5.1.7. Emerging evidence for non-glucosinolate isomers of glucosinolates: "isoglucosinolates"

A computerized system for evaluation of HPLC-MS/MS data of GSL analysis was developed and claimed to be able to identify GSLs (Sun et al., 2016) by a complex procedure taking into account high resolution mass, fragmentation and isotope (^{34}S) pattern. Authentic references were generally not used. In the supplementary data, it can be seen that the program identified multiple peaks as isomers of the same GSL. For example, no less than fifteen chromatographic peaks were assigned as allyl GSL or an isomer (t_R 1.6–6.6 min in a 25 min gradient), five peaks as butenyl GSL or an isomer (t_R 1.7–7.3 min), six peaks as hydroxybut-3-enyl GSL or an isomer (t_R 1.9–4.2 min), ten peaks all identified exactly to phenethyl GSL (t_R 5.6–9.2 min), six peaks all identified exactly as indol-3-ylmethyl GSL (t_R 5.3–7.1 min), four peaks as 4-hydroxyindol-3-ylmethyl GSL or an isomer (t_R 3.0–4.7 min), and seven peaks as isomeric methoxyindol-3-ylmethyl GSLs (t_R 7.8–10.8 min). There was no discussion of t_R in the structural assignments; and, e.g., "allyl GSLs", "butenyl GSLs" and "hydroxybutenyl GSLs" eluted apparently in mixed order, although we notice that t_R values do appear to be located in reasonable zones. There was no discussion of validation using reference materials except the use of 3–4 authentic references, all very polar. The authors concluded that the program "GLS-finder" was useful for interpretation of GSL analytical results. Based on the published data, the present reviewers notice the apparently high ambiguity

of the results. It can be noted that the claimed number of peaks exceeds by far the number of known GSLs for each elemental composition. In some cases, the number of GSL isomers even exceeds the theoretically predictable number. Thus high resolution MS/MS may be a much less specific tool for GSL identification than claimed in the paper. In addition, non-GSL isomers (see below) may complicate matters in some plants. We further conclude that using a range of representative authentic standards or equivalent reference materials is still mandatory for reliable GSL analysis. Another paper from the same group (Shi et al., 2017) further claimed highly surprising structures apparently without comparison with authentic references or reference materials. However, an important result of these papers is the apparently high number of GSL isomers and GSL-like compounds in plant samples when assessed with modern highly sensitive instruments, a theme continued by the next publication discussed.

A recent report (Capriotti et al., 2018) is problematic in terms of structural suggestions, but valuable in terms of analytical optimization and evidence for multiple GSL isomers. No less than six hydroxyl derivatives of indol-3-ylmethyl GSL (**43**) were claimed from cauliflower based on HPLC-HRMS data, along with numerous other claims, without any consideration of t_R or even comparison with known **43** or the known, commonly available hydroxyl derivative **28**. From previous literature, two hydroxyl derivatives are known (**28**, **[154]**) and one unidentified (Cataldi et al., 2007) has been proposed (Fig. 15C). All elute moderately earlier or slightly later (desulfo **[154]**) than the parent compound, **43**, as expected (Cataldi et al., 2007; Pfalz et al., 2016). Comparison with t_R of authentic **43** would hence be highly relevant for the reliability of the proposed hydroxyl derivatives. Furthermore, we are not aware of any species investigated to date where a hydroxyl derivative of **43** accumulates without co-occurrence with **43** itself. However, **43** was apparently not present in the investigated cauliflower (Capriotti et al., 2018). In contrast, the presence of two proposed "cinnamoyl indolyl" GSLs were claimed. Numerous other highly unusual structures were claimed and "identification confidence levels" assigned, such as three "methylsulfinylbutyl GSLs" and two "methylsulfinylpropyl GSLs", eluting in mixed order and all assigned "identification confidence level 2" although only one of each is so far known to exist in nature (Fig. 15G).

Comparing these problematic but also interesting contributions (Sun et al., 2016; Capriotti et al., 2018), there seems to be potential evidence of so many GSL isomers that some of them would seem not to be GSLs by definition (Section 1.1.1.). These isomers could be epimers of the glucose residue or traces of *α*-anomers, glucoside-*O*-sulfates and (*E*)-sulfate GSL stereoisomers. A combination of such isomers, which could form spontaneously in non-enzymatic isomerizations or from less than absolute specificity of biosynthetic enzymes, could explain minute amounts of isomers accompanying the major GSL (Fig. 29). This is a key issue to be taken into account in structure elucidation and studies of GSL biosynthesis and turnover, because previously unknown isomeric structures of what was thought to be the "constant part" of putative GSLs now must be considered. We propose calling these so far hypothetic metabolites "isoGSLs", some of which have been reported as synthetic compounds (Section 3.). The definition would exclude GSLs but include all isomers in the GSL backbone structure. No isoGSL has been described with certainty during more than a century of careful GSL research, so isoGSLs can probably be disregarded in the traditional analytical range, say, at levels typically detectable using HPLC-UV. Hence, we suggest the need to take isoGSLs into account mainly when interpreting minor peaks in HPLC-MS.

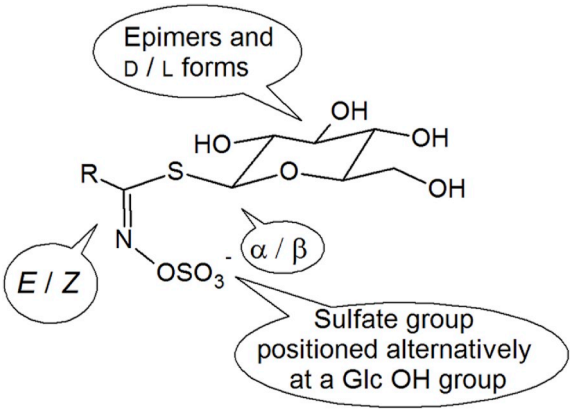


Fig. 29. Possible sites for formation of isomers of the constant part of glucosinolates. Very high numbers of isomers of glucosinolates suggested in two recent publications could potentially be such "isoglucosinolates".

5.1.8. The need for control experiments and presenting uncertainty

We believe that publishing poorly evidenced claims without taking into account previous knowledge and without carrying out even simple control experiments is counterproductive. If such control experiments are not deemed worth the effort, one should consider whether the entire paper is of sufficient interest to be published at all. Reviewers and editors should demand obvious control experiments carried out for testing of any claimed structures, rather than allowing publication of HPLC-MS data sets without further inquiry. Apart from comparison with t_R of relevant authentic GSLs and the obvious relevance of NMR and GC-MS of myrosinase products, inspiration for control experiments are abundant in the historical literature. They include simple chemical derivatization (Cataldi et al., 2007) and collection of HPLC peaks followed by testing of sensitivity to myrosinase (e.g. Kjær and Gmelin, 1957a; Dauvergne et al., 2006; Agerbirk and Olsen, 2012), sulfatase, esterases in the case of proposed acyl derivatives (Reichelt et al., 2002) and glycosidases in the case of proposed glycosides such as **109** (Fig. 15F) (Bennett et al., 2004; Dalby-Brown et al., 2011). Finally, comparison with an authentic reference obtained by rational synthesis (Section 3) can be used even for trace level analytes.

Clearly, presentation of remaining uncertainty is essential. As an example of how to correctly handle uncertainty, Mithen et al. (2010) published HPLC-MS data for a range of species, without the use of authentic standards but with implicit consideration of relative retention times. Consequently, the authors wisely abstained from exact peak identification, but simply named peaks as one of several possible isomers, e.g. hydroxyphenylethyl isomers, hydroxymethylpropyl isomers. Only few unsubstantiated claims were made. A more recent example of transparent presentation of the basis of tentative peak identification is Buckley et al. (2019). Additional examples of specifically pointing out the remaining uncertainty of detected GSL peak identities are many (Agerbirk et al., 2010a; Glauser et al., 2011; Pasini et al., 2012; Lelario et al., 2012; Bianco et al., 2014; Agneta et al., 2014; Förster et al., 2015; Maldini et al., 2017; Montaut et al., 2018).

5.2. GC-MS

For indirect identification of GSLs, products of controlled myrosinase-catalyzed hydrolysis (e.g. Feeny and Rosenberry, 1982; Daxenbichler et al., 1991) are of interest. This is because the products reflect the variable GSL side chain and myrosinase dependency support their GSL origin. The most important kinds of products are ITCs, nitriles (Fig. 22) and/or OATs (Fig. 9). Due to their often apolar and hence volatile nature, many of these products are suitable for GC-MS analysis.

At the end of the 20th century, many authors reported GSLs in different plant species solely based on GC-MS analysis of their autolysis (e.g. Cole, 1976) or controlled myrosinase hydrolysis (e.g. Daxenbichler et al., 1991) products. The first reports of a very large number of plants from Brassicaceae (76), Plantaginaceae (1), and Resedaceae (2) were authored by Cole (1975, 1976, 1980). Later, Daxenbichler et al. (1991) reported an even more comprehensive study of GSLs by GC of their hydrolysis products in seeds from 297 species of wild plants from the

Brassicaceae (259), Capparidaceae (22), Caricaceae (1), Moringaceae (2), Phytolaccaceae (3), Pittosporaceae (1), Resedaceae (6), Salvadoraceae (1), and Tropaeolaceae (2). These publications still represent some of the main reports of GSL distribution among plant species (Fahey et al., 2001). In addition to its use for indirect GSL identification, GC-MS analysis of GSL products is of course also valuable in its own right for analysis of the actual products, and much of the discussion below is also of relevance in this perspective. However, our focus is on GC-MS for indirect GSL identification.

In the following section, reference papers and databases are presented, along with particular analytical aspects of the various structural groups, analytical aspects of some controversial structures, and analytical consequences of instability of some GSL products in complex matrices.

5.2.1. Mass spectral reference data for glucosinolate products

Initial discovery of many naturally-occurring GSLs was done indirectly by identification of ITCs using mass spectrometry (MS). The systematic study of the mass spectra of 40 naturally-occurring as well as synthetic ITCs including alkyl (straight chain and branched), methylsulfanylalkyl, alkenyl, and phenylalkyl was performed by Kjær et al. (1963) and has served as a literature database for identification of GSLs in many plants. Other volatiles, such as nitriles and OATs that can be detected during GC-MS analysis, can help in indirect identification and confirmation of GSLs. Consequently, Spencer and Daxenbichler et al. (1980) reported an additional mass spectra "database" that includes alkyl or phenylalkyl ITCs and nitriles containing various functional groups (methylsulfanyl, methylsulfinyl, methylsulfonyl, episulfide, hydroxyl) and OATs. Even today, GSLs are identified by their degradation products, but with difficulty because the number of their mass spectra is still low in the commercially available databases. Only a limited number of mass spectra of ITCs deriving from naturally-occurring GSLs is available in the "NIST 17" and "Wiley 11" MS databases, such as for **11**, **12**, **[13]**, **15**, **[16]**, **[20]**, **23**, **45**, **46**, **51**, **54**, **[55]**, **[59]**, **61–66**, **88**, **92**, **94**, **95**, **101**, **[102]**, **105**, **107**. Thus, the literature contains some valuable information on the mass spectra of ITCs as well as other volatile GSL degradation products (nitriles, OATs) and GC-MS is still used for indirect GSL identification. However, identification by GC-MS is in many cases based mostly on retention time data and interpretation of recorded MS spectra, without direct comparison to commercially available or synthetically obtained standards. Nitriles originating from the same GSLs are often found at lower retention indices on the commonly used low polarity columns, and their mass spectra and retention indices can support GSL identification. Mass spectra of ITCs, nitriles or others, have been reported by many authors after Kjær et al. (1963) and Spencer and Daxenbichler (1980) and are still the main source used for indirect identification of GSL (Hogge et al., 1988; Gardiner et al., 1999; Songsak and Lockwood, 2002; Al-Gendy and Lockwood, 2003; Vaughn and Berhow, 2005; Radulović et al., 2008; Blažević and Mastelić, 2009). A particular feature of MS recognition of GSL degradation product candidates is the "nitrogen rule": due to the odd number of nitrogen atoms in ITCs as well as nitriles, the mass of the molecular ion (M^+) is odd. For this reason, the N-containing GSL products can be easily distinguished from most other volatiles that are usually found during GC-MS analysis such as acids, alcohols, alkanes, etc. In the cases where M^+ is not visible, chemical ionization (CI) could be used, but this is a rare situation. Al-Gendy and Lockwood (2003) used CI-MS using ammonia under the same operating conditions as for standard electron ionization mass spectrometry (EI-MS, using ionization strength of 70 eV) and the visibility of the molecular ion increased in the cases of 3-(methylsulfinyl)propyl ITC (from 5 to 48%) and 4-(methylsulfinyl)butyl ITC (from 0.1 to 18.5%).

5.2.2. Straight-chain and branched alkyl glucosinolates

Alkyl GSLs with straight chain that are suggested in nature include C_1, C_2, C_4–C_6 GSLs i.e. **51**, **[16]**, **[13]**, **[102]**, and **[20]**, respectively (Fig. 15A). *n*-Propyl ITC, which would be a product of now discontinued "108", has not to our knowledge been reported from plants. GSLs are also reported to contain branched-alkyl chains that are isomers of propyl (**56**), butyl (**61** and **62**), pentyl (**54** and **[55]**), and hexyl (**58** and **[59]**) GSLs (Fig. 15B). Contrary to the GSLs with a straight-alkyl chain, most branched-alkyl GSLs structures (but not **[55]** and **[59]**) have been confirmed by NMR of the intact GSL and/or desulfo counterpart.

In addition to M^+, EI mass spectra will always display many ions stemming from fragmentation. Some of these may often serve to differentiate isomers. Using GC-MS analysis of volatiles for identifying GSLs in horseradish (*A. rusticana*), Grob and Matile (1980) reported i. a. the mixture of butyl and pentyl isomers. One of the most characteristic fragment in mass spectra of ITCs with straight and branched alkyl groups is *m/z* 72 that corresponds to $[CH_2NCS]^+$. However, fragmentation at alkyl branching positions is also favored, so for branched ITCs stable carbocations are often formed in a way allowing differentiation of isomers. For instance, pentyl ITC isomers (M^+, *m/z* 129) which originate from straight chain **[102]** (Fig. 15A), Ile-derived **54** and Leu-derived **[55]** (Fig. 15B) can be easily distinguished. In addition to the *m/z* 72 fragment, 3-methylbutyl ITC from **[55]** forms the fragment *m/z* 114, while 2-methylbutyl ITC from **54** forms the characteristic fragment *m/z* 73 of similar intensity to *m/z* 72 and the fragments *m/z* 100 and *m/z* 114. For that reason, after comparison with published MS spectra (Kjær et al., 1963), Grob and Matile (1980) identified 3-methylbutyl ITC for the first time as naturally-occurring and suggested the presence of **[55]**, although the evidence is still weak (Table 1). In general, the straight-chain alkyl GSLs have never been isolated and their NMR spectra never obtained for unambiguous proof of their existence. Although their ITCs are distinguishable by mass spectra and retention indices from the branched ones, the corresponding GSLs require confirmation by isolation and characterization, e.g. by NMR.

5.2.3. Glucosinolates with an aromatic ring

GC-MS has successfully been used to identify phenylalkyl ITCs with and without substitution on the phenyl ring, suggesting the presence of the corresponding GSLs. Some of these are highly substituted, such as disubstituted **15** and trisubstituted **114** (Fig. 15D) found in the peppergrass (*Lepidium coronopus* (L.) Al-Shehbaz) by identification of the ITCs, i.e. 3,4-dimethoxybenzyl ITC and 3,4,5-trimethoxybenzyl ITC (along with the corresponding nitriles), respectively (Radulović et al., 2008). Pagnotta et al. (2017) further reported GC-MS data of ITCs and non-ITC products from **15**, **22**, **114** and **152** (an analogue of **114**, bearing a *para* hydroxyl group instead of a *para* methoxy group, Fig. 15D). Therefore, as in the case of ITC from **23** (Fig. 8), the ITCs from **22** and **152** were unstable and not detected, so those GSLs may have been missed in GC-MS investigations focusing on ITCs.

Hogge et al. (1988) reported the presence of several benzoyloxyalkyl GSLs in *A. thaliana* using LC-MS and GC-MS techniques. Based on LC-MS identification of the dGSL and comparison with the published mass spectrum of 3-benzoyloxypropyl ITC (Spencer and Daxenbichler, 1980), Hogge et al. (1988) reported the presence of the corresponding **[10]** (Fig. 15I). Furthermore, the analyses by GC-MS showed ITCs with mass spectra and t_R consistent with 4-benzoyloxybutyl- (from **5**), 5-benzoyloxypentyl- (from **117**), and 6-benzoyloxyhexyl ITCs (from **[118]**) (Fig. 15I). Although isomers are possible, and synthetic standards were not used for comparison, the applied logic of MS and t_R by two combined techniques increased certainty of the presence of these GSLs. Two of them were later confirmed (**5** and **117**, Fig. 15I) (Reichelt et al., 2002), while others still need verification.

Using a "DB-5MS" column in GC-MS analysis and more extreme oven programming (70–290 °C at a heating rate of 5 °C/min), Radulović et al. (2014) reported t_R and mass spectra of 2-(α-L-rhamnopyranosyloxy)benzyl ITC (M^+, *m/z* 311), which derives from **109** present in *Reseda lutea* L. (Fig. 15F). This ITC was isolated and confirmed with spectroscopic analyses (IR, 1D and 2D NMR). The GSL was previously

known, but direct detection of this type of hydrophilic ITC by GC-MS represents an extension of the use of GC-MS in GSL product analysis.

5.2.4. Discontinued and uncertain glucosinolates

4-Oxopentyl GSL (discontinued "99", Table 1) was discontinued by Agerbirk and Olsen (2012) since the claimed literature report did not exist. A later tentative identification of the corresponding ITC in *Erysimum corinthium* (Boiss.) Wettst (Al-Gendy et al., 2010) without structural discussion was not found to be a sufficient basis for re-introducing this as a suggested GSL. Indeed, the reported mass spectrum did not contain the fragment $m/z = 72$, which should be present if the compound was the suggested ITC.

Another formerly listed GSL that was discontinued by Agerbirk and Olsen (2012) was phenyl GSL, **[103]**, based on weak evidence: detection of the ITC as a trace peak by GC-MS among volatile products in brown mustard (*Brassica juncea* (L.) Czern.) leaves and horseradish (Kojima et al., 1973). However, this ITC was also identified (but not consistently) in *F. aegyptia* volatiles by co-chromatography with an authentic standard (Al-Gendy and Lockwood, 2003). Since the present review has adopted the category of partially characterized GSLs in brackets, **[103]** was re-introduced in brackets, although it is still uncertain whether rarely detected phenyl ITC is derived from the corresponding GSL **[103]** or is formed in other ways in plant extracts. The fact that phenyl ITC was not detected in the extensive screenings by Cole (1975, 1976), Daxenbichler et al. (1991) or in the systematic investigations by Kjær and coworkers, suggests that formation of phenyl ITC in plants is very rare and needs confirmation.

5.2.5. Analytical challenges caused by instability

Critical aspects that should be considered in the practical use of GC-MS for GSL identification include the reactivity and stability of ITCs. As ITCs contain a quite electrophilic carbon atom, they tend to react with biological nucleophiles (hydroxyl, amino, or thiol groups) to form *O*-thiocarbamates, thiourea derivatives, or dithiocarbamates, respectively (reviewed by Bones and Rossiter, 2006; Hanschen et al., 2014) (Section 4.4.). A well-investigated case is allyl ITC (Chen and Ho, 1998). Some ITCs with substituents activating a benzylic position are prone to fast hydrolysis, and are generally not detectable by GC (De Nicola et al., 2013b; Pagnotta et al., 2017) (Fig. 8). This problem includes the indole GSLs, for which ITC products are thus not detectable with GC. However, nitriles from these GSLs are more stable. For instance, the corresponding nitrile of indol-3-ylmethyl GSL (**43**, Fig. 15C) can be determined by GC-MS (e.g. Songsak and Lockwood, 2002, 2004).

Despite the alleged "high" reactivity of ITCs in biological matrices, they may survive at appreciable levels for a long time in complex matrices such as blood (Holst and Williamson, 2004; Angelino and Jeffery, 2014) and insect frass (Schramm et al., 2012). This is possibly due to the reversible nature of the reaction with thiols, the lack of nucleophilicity of amines at near neutral pH, and the moderate nucleophilicity of water and alcohols (Hanschen et al., 2014) (Section 4.4.). Quantification of adducts of benzyl-, phenylethyl- and 4-rhamnosyloxybenzyl ITCs applied to or formed in plant matrices and subject to prolonged incubation revealed that only a minor amount of the applied ITC was converted into dithiocarbamate, while the major amount was found as intact ITC or lost by evaporation, depending on volatility (Müller et al., 2015, 2018; Agerbirk et al., 2015b). Indeed, semi-quantitative extraction and subsequent determination of ITCs from plant matrices is realistic but not simple (e.g. Lambrix et al., 2001; Witzel et al., 2013). Determination of recovery or use of a representative ITC as internal standard is recommendable, to take into account loss of the semistable ITCs. Measurement of recovery of the actual ITC would seem preferable as different GSL-derived ITCs showed different rates of reaction in realistic model systems (Hanschen et al., 2012; Luang-In and Rossiter, 2015). One paper reported around 50% of recovered ITC (Lambrix et al., 2001).

Nitriles seem generally to be inherently more stable than ITCs (Hanschen et al., 2014; Luang-In and Rossiter, 2015), although fast enzymatic turnover can happen when nitrilases are present (Section 4.4.).

A special analytical problem in GC-MS is thermal instability of some GSL products. Mass spectra of ITCs originating from C_3–C_5 alkenyl GSLs (**107**, **12**, and **101**, Fig. 15I), the existence of which is ascertained by NMR data, are available in commercial databases. However, but-3-enyl ITC has also been reported to be a thermal degradation product of 4-(methylsulfinyl)butyl ITC (originating from **64**, Fig. 15G) caused by the high temperature used in the injection ports of GC and GC-MS (Chiang et al., 1998). Thus, interpretation of observed ω-alkenyl ITCs in GC-MS is not straightforward, as they can be indicative of the directly corresponding GSLs as well as the corresponding ω-(methylsulfinyl)alkyl GSLs.

Indeed, higher homologs of alkenyl ITCs may be artefacts. Although C_6 and C_7 alkenyl GSLs were previously characterized on the basis of their GC-MS reports, hept-6-enyl- (previously "18") and hex-5-enyl GSLs (previously "19") were discontinued by Agerbirk and Olsen (2012). Songsak and Lockwood (2002) suggested the presence of C_8–C_{10} alkenyl GSLs in *Nasturtium montanum* = *Rorippa indica* (L.) Hiern by reporting only mass spectra of their corresponding ITCs. A later study by GC-MS analyses of volatiles from tower rock-cress (*Arabis turrita* L.) revealed also C_7–C_{11} alkenyl GSLs by their ITCs and/or nitriles, but the parallel analysis by LC-MS did not detect their presence (Blažević et al., 2015). It was suggested that these volatiles are thermal artefacts formed during GC-MS analysis from the long-chain thio-functionalized GSLs that were confirmed by LC-MS. Thus, we did not include these GSLs in Table 1, as the evidences suggest that GC-MS is not an appropriate analytical tool for long-chain alkenyl GSLs, and they need verification by other analytical tools such as NMR.

The stability of epithionitriles is poorly known, but the one from allyl GSL (**107**, Fig. 15I) is particularly unstable, forming 2-aminothiophene at room temperature and further products after heating (Hanschen et al., 2018a).

Finally, ITCs bearing a *β*-hydroxyl group are unstable and spontaneously cyclize, producing OATs (Fig. 9), which show an intense molecular ion. OATs were reported using GC-MS analysis of various plant extracts (Gardiner et al., 1999; Songsak and Lockwood, 2002; Radulović et al., 2017). However, due to poor sensitivity the use of HPLC-UV was recommended as the preferable analysis method (Wathelet et al., 2004). Thermally induced rearrangement to isomeric thiazolidin-2-ones during GC is also well-established (Lutfullin et al., 1976; Radulović et al., 2017). Finally, OATs are unstable in some crushed plant tissue due to enzymatic exchange of the sulfur with oxygen in a yet poorly understood reaction (Fig. 27G).

6. Perspectives

Major goals of GSL research are identification of genetic resources and biochemistry for specific GSLs, as well as elucidation of biological roles and structure-activity relationships for GSLs. In all cases, correct identification of GSLs is of paramount importance. Since reliable GSL identification has been neglected in parts of the literature in recent years, we hope that the recommendations in the present review will stimulate attention to quality. We also hope that the review of GSL degradation and their organic synthesis will promote biochemical and structure-property investigations.

In future tentative GSL identifications, explicit discussion of remaining uncertainty should be a priority in order to avoid misunderstandings, and we stress the importance of carrying out more control experiments to improve the reliability of results. Editors and reviewers share a huge responsibility in that respect.

More common use of authentic standards and reference materials is another priority that is apparent from this review. It will be a mixed responsibility of new-coming groups to acquire such materials, and of established groups to provide such materials in reasonable ways.

Finally, we hope that our overview of currently missing evidence and remaining uncertainty within the GSL literature will stimulate researchers to re-investigate questionable cases, in order to consolidate and expand the scientific understandings of GSL diversity.

7. Experimental

7.1. Isolation of **141**

DEAE-Sephadex A-25 (1.5 g) was swelled overnight in an acetate buffer (0.5 M, pH 5.0) (prepared by titrating 0.5 M aq. AcOH with NaOH to ensure acetate being the only anion) and distributed to five mini-columns, which were washed with 5 × 2 mL H_2O each. *Cardamine pratensis* seeds (Product No. 272F) were from Chiltern Seeds (Ulverston, UK). Jelitto Perennial Seeds (Schwarmstedt, Germany), were indicated as the primary source of the seeds. *C. pratensis* plants were grown from seeds in a greenhouse, and foliage harvested from first year rosette plants. This batch of foliage was dominated by **141** (with ca. 1% of **43**) as measured by the dGSL procedure, in contrast to a previously analyzed batch with significant levels of **43** (Agerbirk et al., 2010a). Freeze-dried foliage (5.0 g) was extracted in 200 mL of boiling MeOH–H_2O (1:1) for 2 min on a hot-plate (solvent first brought to boiling, then poured over the foliage). The extraction was repeated with fresh solvent. The total crude extract was distributed evenly over the five mini-columns. Then, each column was washed with 4 mL extraction solvent, 12 mL AcOH-EtOH-H_2O (1:1:3) (in order to elute weak acids) and 4 mL H_2O, and eluates were discarded. Subsequently, **141** was eluted from each column with 6 × 2 mL of sat. K_2SO_4 in H_2O–EtOH (19:1) (approximately 0.5 M K_2SO_4), collecting each 2 mL fraction in a centrifuge tube and waiting 1 min between each 2 mL to ensure equilibration. The fractions were numbered and kept separate. In order to remove most of the K_2SO_4, 4 mL of cold 96% EtOH was added to each 2 mL fraction, followed by centrifugation (2 min, 4000 *g*). Supernatants were analyzed by HPLC of intact GSLs (Agerbirk et al., 2014) (samples diluted 10 x in H_2O before injection). Elution of **43** was delayed, giving partial separation of **141** and **43**. Supernatants rich in **141** (No. 2, 3 (and 4) from each mini-column) were pooled (one pool for each mini-column) and left under a gentle air stream overnight for evaporation, followed by freezing and freeze-drying. To get rid of remaining K_2SO_4 in the solid remnant, **141** was extracted with 3 × 2 mL of hot 100% MeOH from each mini-column batch, leaving methanol-insoluble K_2SO_4 crystals behind. The solvent was allowed to evaporate, and the remaining **141** (K^+ salt) was freeze-dried and weighed (94 mg) (corrected for KAc: 57 mg corresponding to 1.1% of leaf dry wt.). NMR and ion trap MS was carried out as described (Agerbirk et al., 2014), using dioxane as int. std. in NMR (δ_H = 3.75 ppm, δ_C = 67.40 ppm). Contamination of **141** with acetate (Section 7.2.) suggested that significantly more crude extract should ideally have been loaded to each mini-column in order to saturate the matrix with **141**, exchanging acetate. This entire procedure is generally applicable to extracts of both seeds and vegetative plant parts and is a simplified, downscaled adaptation of a procedure by Thies (1988).

7.2. Potassium 3-(hydroxymethyl)pentylglucosinolate

Pale (slightly yellowish) amorphous solid. Ion trap MS: *m/z* 418 $[M-H]^-$. ^{1}H NMR (400 MHz, D_2O): δ, see Fig. 13A, coupling constants and multiplicities as for **d141** (Agerbirk et al., 2010a). ^{13}C NMR (100.6 MHz, D_2O): δ 165.7 (0), 82.6 (1′), 81.0 (5′), 77.9 (3′), 72.8 (2′), 69.9 (4′), 64.4* ($\underline{C}H_2OH$), 61.4 (6′), 41.3 (3), 30.6* (1), 28.5* (2), 23.3* (4), 11.1 (5). HSQC supported assignments, signals labeled * were also confirmed to be $-CH_2-$ by DEPT-135. COSY: See Fig. 13B. HMBC confirmed connectivity and assignment of carbon 0. A three-fold molar impurity of potassium acetate (39% by mass) was revealed by ^{1}H NMR: δ 1.9 (s) (Fig. 13A) and ^{13}C NMR: δ 182.2, 24.2. All NMR spectra are shown in Supplementary Fig. S2.

Conflict of interest

The authors declare to have no patent-related or other financial interests in particular GSLs being generally accepted as natural or not, or in standards, reference materials, equipment, methods or any other aspect of GSL analysis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2019.112100.

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